ANTAGONISTS OF SPONTANEOUS DISCHARGE IN THE DESHEATHED FROG NERVE

by

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Abstract

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These investigations examined the effects of selective antagonists of calcium deficient spontaneous discharge on the desheathed frog sciatic nerve. Selective antagonists are agents which abolish spontaneous discharge at a time when the evoked spike is unaffected. Compounds which depress both spontaneous discharge and the action potential are nonselective antagonists. The effects of selective antagonists were studied on the following parameters: spontaneous discharge, the action potential amplitude, demarcation potential, Ca-45 efflux and ATP content. The purpose of these experiments was to study the mechanism by which a nerve becomes spontaneously active thereby clarifying the role of calcium in excitation. The ability of divalent cations, hydrogen ion, tetrodotoxin, and metabolic inhibitors to substitute for calcium was examined.

I Divalent Cations

The divalent cations tested antagonized spontaneous discharge in

the following order of effectiveness: Ca>Mn>Co>Ni>Zn>Mg, Sr>Ba. Of these cations the best antagonists of spontaneous discharge have ionic radii smaller than but most similar to calcium. The best antagonists of spontaneous discharge (Mn and Co) bind more strongly to nitrogen, sulfur and oxygen ligands than do the other cations. It is proposed that the ability of a cation to antagonize spontaneous activity is related to its ability to bind tightly to calcium binding sites. Demarcation potential measurements indicate that the cations which antagonize spontaneous discharge also repolarize the calcium deficient nerve suggesting a correlation between these two parameters. Data from Ca-45 efflux studies show that cations which are the best calcium releasers are not the best antagonists of spontaneous discharge. Strontium and barium, comparatively poor antagonists, are the most potent calcium releasers. The lack of a correlation between these two parameters is probably due to the difficulty in detecting the fraction of calcium involved in spontaneous discharge. A 25 minute exposure to calcium-free medium reduces the ATP content in the nerves tested. The decrease in ATP is not due solely to the depolarizing action of low calcium medium since exposure to 3 mM KCl Ringer's solution (which causes a similar depolarization) does not decrease the ATP content. The addition of 1.8 mM $MnCl_2$ to calcium-free medium did not prevent the decrease in ATP content caused by calcium deficiency. $SrCl_2$ (1.8 mM) in calcium-free solution further reduced the ATP content of nerves. This action may be related to the calcium releasing ability of strontium.

II Hydrogen Ion

Experiments performed to test the effects of changes in hydrogen ion concentration on spontaneous discharge and the evoked spike indicated that spontaneous discharge was more sensitive to low pH than was the action potential. At a pH between 4.5 - 5.5 spontaneous discharge is completely abolished while the evoked spike is only slightly affected. Antagonism of spontaneous discharge by hydrogen ions may involve the binding of hydrogen to negative membrane sites which normally bind calcium. Ca-45 efflux studies indicate that pH 4.5 does not release calcium from the nerve.

III Tetrodotoxin and Choline Chloride Ringer's Solution

Tetrodotoxin (TTX) (10^{-9}gm/ml) suppressed spontaneous discharge without affecting the evoked spike. The antagonism of spontaneous discharge by TTX is probably due to suppression of the early transient currents necessary for excitation. TTX, whose cationic form is the active form, may be acting on membrane sites which normally bind calcium. Experiments with choline chloride Ringer's solution confirmed that sodium ions were needed to carry the current.

IV Metabolic Inhibitors

Metabolic inhibitors which selectively antagonized spontaneous discharge were: 2,4 dinitrophenol (DNP), potassium cyanide, m-nitrophenol, sodium azide (NaAz), 1,5 difluoro 2,4 dinitrobenzene (FFDNB), 2,4 dinitrofluorobenzene (FDNB), m-fluornitrobenzene (FNB). These

agents act either through a metabolic effect or a direct interaction with important axoplasmic or membrane sites. The findings that all these chemically different inhibitors are effective antagonists and that their time course of action is slow (25-30 minutes) as compared to other agents (TTX and divalent cations) indicated that they are not acting on membrane sites. They most likely affect metabolic pathways, which in turn influence excitability. Demarcation potential measurements showed that m-nitrophenol, DNP and FDNB decreased the ATP content in nerve during the time period that spontaneous activity is abolished.

In summary, a number of different agents which antagonize spontaneous discharge before the evoked spike is affected were studied in desheathed nerves. The divalent cations tested were all capable of antagonizing calcium deficient spontaneous discharge although some were more effective than others. A correlation exists between their ionic radii and their effectiveness as antagonists. Spontaneous discharge is more sensitive to TTX and hydrogen ion concentration than the evoked spike. Experiments with metabolic inhibitors indicate that there may be a link between metabolism and excitability although the mechanism is still obscure.

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INTRODUCTION

It is a widely accepted view that calcium plays a major role in nerve function. The following investigations were designed to learn more about the role of calcium in the excitable cell. High external calcium generally stabilizes nerves while low calcium increases excitability by decreasing the threshold, accomodation and membrane resistance often leading to spontaneous repetitive discharge (Brink, 1954). Spontaneous activity has been observed in squid axons (Arvanitaki, 1939), lobster axons (Dalton, 1958) and frog sciatic nerves (Brink, 1954). The mechanism of this increased excitability is not known. The study of compounds which block calcium-deficient spontaneous discharge would further elucidate calcium's role in conduction. Therefore, agents which selectively antagonize spontaneous discharge in the desheathed frog sciatic nerve at a time when the amplitude of the evoked spike is unaffected were examined. The effects of divalent cations (Mn, Co, Ni, Zn, Mg, Ba, Sr), tetrodotoxin (TTX), hydrogen ion and metabolic inhibitors (2,4 dinitrophenol — DNP —, azide, cyanide, m-nitrophenol, 1,5 difluoro-2,4 dinitrobenzene — FFDNB — 2,4 dinitrofluorobenzene, m-fluornitrobenzene) were investigated on the following parameters: spontaneous discharge, the amplitude of the evoked action potential, the demarcation potential*, Ca-45 efflux and ATP content were

* The demarcation potential is a measurement of the potential gradient observed when two electrodes are placed on different portions of an isolated tissue. Thus, if one electrode is placed on a portion of nerve treated with a depolarizing solution while the other electrode is placed

also studied in order to further elucidate their mechanism of action on the calcium deficient nerve.

It has been suggested that calcium and hydrogen ions bind to the same negative membrane sites (Hafemann, 1969). Experiments were performed to determine whether spontaneous discharge was more sensitive to changes in hydrogen ion concentration than was the evoked spike and, if so, to find the pKa of the calcium binding sites involved in spontaneous discharge.

TTX blocks the current through the early transient channel in a variety of nerves. Attempts were made to examine whether low concentrations of TTX could block spontaneous discharge without affecting the evoked spike. A difference in sensitivity to TTX may indicate that different channels or a fewer number are involved in the initiation of spontaneous discharge. Nerves exposed to sodium-free choline chloride Ringer's solution were also studied to examine the role of sodium in spontaneous discharge.

The role of metabolism in nerve excitation is still obscure. Gerard (1932) proposed that calcium links metabolic processes to excitation in nerves. The determination of whether metabolic changes initiate and/or accompany spontaneous discharge would have a direct bearing on the question of a linkage between metabolism and excitation and on the possible role of calcium. The ability of a variety of metabolic inhibitors to selectively

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on a portion of nerve in normal Ringer's solution a potential difference can be recorded. Because of short circuiting between the two electrodes the demarcation potential is considerably lower than the real potential between the two segments of nerve.

antagonize spontaneous discharge was studied since it has previously been reported that DNP and azide antagonize spontaneous discharge (Altura, 1968). The effects of these agents on ATP content, demarcation potential and Ca-45 efflux were also investigated.

The frog sciatic nerve contains about 3,000 - 4,000 myelinated fibers and about twice as many nonmyelinated fibers. The fiber size ranges from 3 to 29 microns.

The desheathed nerve was used in these experiments because it has been shown that the sheath acts as a permeability barrier to electrolytes and other compounds (Krnjevic, 1954). Spontaneous discharge appears in the desheathed frog nerve within 30 seconds after exposure to calcium-free medium while it takes 3-4 hours to develop in the sheathed nerve (Altura, 1968).

In summary, antagonists of spontaneous discharge were studied in order to better understand the mechanism by which a cell spontaneously discharges, thereby elucidating the role of calcium in stabilizing the nerve.

HISTORICAL BACKGROUND

Calcium Concentration and Nerve Function

The importance of calcium in the maintenance of excitability was first recognized by Ringer (1883) who found that calcium was needed to prevent cessation of the beat in excised frog heart. Generally an increase in the calcium concentration in the medium stabilizes the nerve whereas a decrease of the calcium concentration causes the nerve to become more excitable (Shanes, 1958). Decreasing the calcium concentration lowers membrane resistance (Cole, 1949), reduces accomodation (Solandt, 1936) and decreases threshold (Brink, et al., 1946).

Reducing the calcium concentration also causes local oscillatory behavior (recorded with external electrodes) whose amplitude is inversely proportional to the calcium concentration. Oscillatory behavior eventually gives rise to spontaneous discharge, the frequency of the discharge being related to the frequency of the local oscillations (Brink and Bronk, 1941). Stampfli and Nishi (1956) using the sucrose gap technique found that bundles of myelinated frog nerves depolarized by 8 to 10 millivolts in calcium-free medium. Giant axons sometimes show a slight depolarization in low calcium medium (Shanes, 1958). In caclium-free medium both the squid axon (Frankenhauser and Hodgkin, 1957) and the isolated frog node of Ranvier (Frankenhauser, 1957) become inexcitable. The whole desheathed frog nerve can maintain its excitability in calcium-free medium (Frankenhauser, 1957).

The effects of calcium concentration on potassium and sodium permeabilities in voltaged clamped squid axons were studied by Frankenhauser and Hodgkin (1957). Decreasing the calcium concentration caused the sodium current entering the axon during a moderate depolarization to increase although the maximum sodium conductance attained for a large depolarization was not affected. The rate at which the sodium conductance rose and the amount of sodium conductance which was inactivated were also increased. The rate of rise and magnitude of the potassium conductance were increased by lowering the calcium concentration. Hille (1968) studying the voltage clamped frog node of Ranvier found changes in the potassium and sodium conductances similar to those in squid axons as a result of changing the calcium concentration.

Calcium Binding to Membranes

It is a widely accepted view that calcium binding to negative membrane sites influences membrane permeability and therefore excitation (Gordon and Welsh, 1945; Tobias, 1964). Negative charges on the cell membrane surface have been demonstrated in red blood cells (Abramson, et al., 1942), nerve and other cells (Elul, 1967). Bolingbroke and Maizels (1959) have shown that red blood cell membranes bind calcium and recent electrophoretic studies indicate that calcium is bound to erythrocyte and leucocyte membranes at two different anionic sites (Seaman, et al., 1969). Microincineration studies demonstrated the presence of calcium in the myelin of nerve bundles (Scott, 1940).

Support for the hypothesis of calcium binding to nerve membrane

comes from experiments using membrane fractions and artificial membranes. Lipid and lipoprotein membrane fractions from frog skeletal muscle were shown to bind calcium (Koketsu, et al., 1964). Experiments with artificial membranes have shown that calcium binds to monolayers of lecithin (Koketsu and Kimizuka, 1962), phosphatidylserine and phosphatidylethanolamine (Tobias, 1964). Calcium binding to lecithin monolayers is saturated at physiological calcium concentrations and sodium, hydrogen and potassium compete with calcium for binding sites on the lecithin monolayer (Koketsu and Kimizuka, 1962). The binding of calcium to these phospholipids present in nerve membranes may be important in excitation (Tobias, 1964).

Calcium binds to proteins (Klotz, 1946). Several lines of evidence indicate that proteins are important in excitation. Injections of proteases into the squid axon cause it to become inexcitable without affecting the resting potential (Rojas and Luxoro, 1963). Hunneaus-Cox, et al., (1966) found that sulfhydryl reagents which most likely react with protein side chains cause the axon to become inexcitable. ATPase is located on the nerve membrane (Canessa-Fischer, et al., 1967) and Tobias (1964) has suggested that its function may be related to excitability. A calcium sensntive ATPase has been found in red blood cell membranes (Seaman, 1969). Nerve membrane ATPase may function in excitation through the breakdown of a Ca-ATP complex as suggested by Abood and Gabel (1965).

Calcium Flux Measurements in Nerve

It is possible that different fractions of the total cell calcium have different physiological functions and that only one calcium fraction is involved in excitation. For this reason a number of attempts have been made to identify different fractions of cell calcium. Tipton (1934) measured the calcium content in frog nerves under a variety of conditions and concluded that only 40 per cent of the total calcium in nerves is exchangeable. Radiocalcium studies in crab nerve and squid axons indicate that the exchangeable calcium fraction may be divided into two groups: the extracellular fraction with a very fast half time and the intracellular compartment with a slow half time of efflux (Soloway, et al., 1953; Hodgkin and Keynes, 1957). The inexchangeable fraction and two exchangeable fractions have also been identified in muscle (Gilbert and Fell, 1957). Shanes and Bianchi (1959) found that the exchangeable axoplasmic fraction in muscle could be divided into the fraction which exchanges only with calcium (self-exchangeable fraction) and a second fraction which will exchange with sodium, potassium and hydrogen. Presumably it is this second fraction which is lost in calcium-free medium and therefore is linked to spontaneous discharge.

Hodgkin and Keynes (1957) investigating the movements of radioactive calcium in the squid axon found that most of the axoplasmic calcium was bound. They also noted a significant increase in the influx of calcium during stimulation with no parallel increase in calcium efflux. Koketsu and Myamoto (1961) did find an increase in the calcium efflux during stimulation in the frog nerve.

Role of Calcium in Excitation

Calcium has been postulated to influence membrane permeability and excitation. This cation may plug pores in the membrane (Mullins, 1962), structurally modify the membrane (Tobias, 1964), or change the surface potential of the membrane (Frankenhauser and Hodgkin, 1957). Gordon and Welsh (1945) suggest that calcium stabilizes the membrane by linking adjacent polar groups on the axon's surface. Mullins (1961) proposes pores or channels 4 A^{o} in diameter which are blocked by calcium in the resting axon. This investigator further postulated that during excitation calcium is removed from these pores and sodium enters. Adelman (1960) suggested that the pores can vary in size thereby discriminating between potassium and sodium. Alternatively, Chandler, et al., (1965) think there may be completely different channels for potassium and sodium.

Recent studies measuring fluorescent changes in crab nerves stained with acridine orange indicate that there are major physiochemical changes occurring in the nerve membrane during conduction (Tasaki, et al., 1969). Experiments by Cohen and Keynes (1968) on the optical properties of the crab nerve also demonstrate changes in membrane configuration during excitation. Tobias (1964) proposes that changes in the protein structure of the membrane are involved in excitation. He postulates that the outward flux of potassium from the nerve displaces calcium from membrane sites. This displacement leads to an increased hydration of the membrane and a change in protein configuration. These changes increase the permeability of the membrane thus allowing excitation to occur. Experiments with monolayers of phosphatidylserine

indicate that the increased resistance caused by calcium in these monolayers may be due to a decrease in hydration (Tobias, 1964).

Tasaki and Singer (1966) propose that the membrane can maintain two stable states. The first is the calcium dissociated state and the second is the calcium associated state. Excitation occurs during the transition from one state to the other and depends on the rate of dissociation and association of membrane calcium rather than the influx of a monovalent cation. Experiments on the perfused squid axon (Tasaki, et al., 1966) indicate that excitability can be maintained in the absence of external monovalent cations.

It is also postulated that the effects of calcium may be explained by an effect on the surface potential of the nerve fiber (Frankenhauser and Hodgkin, 1957). If the outer boundary has fixed negative charges the concentration of calcium bound to these charges will influence the number of negative sites which are covered by calcium and the surface potential will therefore become more positive. The membrane would behave as if it were hyperpolarized, just as axons behave in high calcium.

All the theories discussed here to explain the stabilizing action of clacium on nerve involve the binding of calcium to membrane sites. Whether this binding exerts its influence by controlling the surface potential, the physical configuration of the membrane or permeability through membrane pores has not been decided. None of these theories are mutually exclusive and it is possible that a combination of these factors is operating.

A great deal of evidence has recently accumulated indicating that calcium has an action on excitable systems other than its stabilizing

effect. Calcium has been shown to be capable of carrying the current in frog sympathetic ganglion cells (Koketsu and Nishi, 1969) and squid giant axons (Tasaki, et al., 1966) when they were placed in isotonic $CaCl_2$ solution. The peak of the action potential was proportional to the external calcium concentration. It is possible that calcium is acting as a charge carrier even in solutions containing sodium ions. The calcium influx has been shown to increase during the action potential in the squid axon (Hodgkin and Keynes, 1955) and may be making a small contribution to the charge entering the nerve.

Effects of Different Agents on Nerve Excitability

A. Divalent Cations

The ability of divalent cations to substitute for calcium has been tested. By measuring the demarcation potential in crab nerve Guttman (1939) found that barium and strontium can counteract the depolarization caused by potassium as does calcium. The order of effectiveness is barium > strontium > calcium. Magnesium has no effect. Barium acts like "high" calcium in the voltage clamped lobster axon while magnesium is much less effective than calcium (Blaustein and Goldman, 1968). Hafemann (1969) has tested the ability of a number of divalent cations to maintain the rate of rise of the action potential in lobster axons. Alkali earth metals are the best calcium substitutes. The transition metals are effective in maintaining the rate of rise of the action potential but the axons become inexcitable within half an hour. Transition metals also raise the threshold of excitability in the lobster axon.

Takahashi, et al., (1962) showed that nickel and cobalt increased the threshold and spike height in calcium deficient amphibian frog nerve fibers. These cations caused a prolongation of the action potential which could be inhibited by increasing the calcium concentration. Nickel is approximately 5 times as effective as calcium in changing the voltage current curves in the frog node of Ranvier (Hille, 1968). Solutions of isotonic $SrCl_2$ and $BaCl_2$ have recently been shown to be able to substitute for isotonic $CaCl_2$ in maintaining excitability in bullfrog spinal ganglion cells (Koketsu and Nishi, 1969). Cells placed in isotonic $MgCl_2$ are inexcitable.

In summary, barium and strontium are generally very effective calcium substitutes while magnesium is not. Nickel, cobalt and other transition metals are able to substitute for calcium but cannot maintain excitability as well as the alkali earth metals in lobster axon. Nickel and cobalt also increase the threshold of frog and lobster nerves.

B. Hydrogen Ion

The effects of changes in hydrogen ion concentration on nerves have been investigated in both the frog node of Ranvier (Hille, 1968a) and the lobster axon (Hafemann, 1969b). A decrease in the pH below 6.0 in the frog nerve results in a decrease in sodium conductance and a shift in the voltage current curves in a way similar to that seen in high calcium. Hille (1968a) explains these results by postulating a block of the sodium channel at low pH values due to the protonation of an acidic group with a pKa of about 5.2. Hafemann (1969b) reports that low pH

causes a reversible deterioration of excitability in the lobster axon without affecting the resting potential. High pH, on the other hand, causes an irreversible disruption of the axon. Increasing the calcium concentration protects the axons from the effects of low pH. Hafemann (1969b), therefore, concluded that calcium and hydrogen are acting on the same membrane sites. Stephens (1969) postulates that a change in the hydrogen ion concentration during depolarization results in the displacement of calcium from the membrane which in turn leads to excitation.

C. Tetrodotoxin

The action of TTX has been extensively studied in a number of nerve preparations. It selectively abolishes the transient inward current in the squid axon (Moore, et al., 1967), the lobster axon (Narahashi, et al., 1964) and the node of Ranvier of frog myelinated nerve (Hille, 1968b). TTX is active in its cationic form (Hille, 1968b; Narahashi, et al., 1969) and it is thought to act by blocking negative membrane sites which control the permeability of sodium. Recent studies on the interaction of TTX with isolated lipids from the membrane of squid axons indicate that TTX reacts specifically with cholesterol (Villegas, et al., 1970). It is therefore postulated that cholesterol is a part of the sodium channel. The action of TTX on the lobster axon is not affected by changes in external calcium concentration (Blaustein and Goldman, 1968).

Metabolism and Excitation

Numerous studies have been carried out to examine the relationship

between nerve excitation and metabolism. The only direct link between excitation and metabolism that has been established is the existence of a metabolically driven Na-K pump which maintains the resting membrane potential. Hodgkin and Keynes (1955) showed that the resting efflux of sodium and influx of potassium was reduced to less than 10 per cent of their normal values by DNP, azide and cyanide. The need for high energy phosphates to maintain these fluxes is clearly demonstrated in perfused squid axons which were blocked by metabolic inhibitors where injection of ATP restored the resting efflux of sodium and influx of potassium (Caldwell, et al., 1960). Post-tetanic hyperpolarization can be inhibited by cyanide, azide, antimycin A, deoxy-D glucose and low temperature (den Hertog and Ritchie, 1969; Greengard and Straub, 1962).

Changes in nerve metabolism take place during activity. Decarboxylation of pyruvate by guinea pig sciatic nerves increases during stimulation. The glucose consumption and lactate production do not significantly change so that the energy needed for the increased activity must come via the Krebs cycle (Reich, et al., 1967). This is in agreement with Brink's (1954) suggestion that there is a shift in intermediary metabolism of glucose to a more complete exidation through the Krebs cycle during stimulation. A decrease in the ATP content of rabbit C fibers after stimulation was reported by Greengard and Straub (1959). No decrease in ATP content was found in frog nerves after stimulation (Cheng, 1961). The difficulty in demonstrating a decrease in ATP in myelinated nerves is probably related to the small change in sodium concentration per impulse as compared with non-myelinated fibers; in the latter the change in sodium concentration per

impulse is much greater.

Calcium and Nerve Metabolism

Gerard (1932) proposed that calcium may link metabolic and electrochemical processes in nerves. A decrease in calcium concentration increases the oxygen consumption in frog nerve fibers (Brink, et al., 1946). This increased oxygen consumption is proportional to the decrease in threshold seen in low calcium Ringer's solution. An increase in oxygen consumption occurs in low calcium medium even in the absence of spontaneous activity (Brink, 1954). It is likely that low calcium is acting by increasing the rate of mitochondrial oxidation since the machinery for oxidative phosphorylation in the nerve is located in mitochondria. Siekevitz and Potter (1953) propose that low calcium increases the rate of oxygen consumption and therefore the rate of oxidative phosphorylation in mitochondria by regulating the concentration of ATP, ADP and AMP.

In calcium deficient nerve there is an increase in the efflux of P^{32} from excitable tissue (Abood, et al., 1962). Abood (1966) has suggested that ATP, calcium and phospholipids form a macromolecular complex on the membrane. During excitation calcium is released and ATP is hydrolyzed thus affecting the membrane configuration and allowing excitation to occur. In this hypothesis the role of metabolic processes in controlling ATP levels in nerves would be intimately associated with excitability.

It has been suggested that changes in the level of free calcium within the axon may influence metabolism (Baker and Blaustein, 1968). The influx of calcium into squid and crab axons is dependent on the sodium

concentration in the axoplasm. Small changes in the sodium content of the axoplasm during excitation may provide significant changes in the level of free calcium inside the cell.

Recent evidence indicates that calcium is extruded by cells. The squid axon and many other cells have a low calcium content (10^{-5}M) indicating that calcium is not distributed according to the Donnan equilibrium (Blaustein and Hodgkin, 1969). Vincenzi and Schatzman (1967) found that red blood cell membranes actively extrude calcium and that ATP probably provides the energy. There is no direct evidence that calcium is actively extruded in the squid axon although the possibility cannot be excluded (Blaustein and Hodgkin, 1969). Baker, et al. (1969), studying the influx of calcium into the squid axon found that the influx of one calcium ion is linked to the efflux of two or three sodium ions.

Metabolic Inhibitors and Nerve Function

The action of metabolic inhibitors on nerves has been studied in order to clarify the role of metabolism in nerve function. 2,4 Dinitrophenol (DNP) causes a slight depolarization in vertebrate fibers at pH 6.0. (Experiments at higher pH values showed no effect, presumably because DNP is more permeable at pH 6.0.) DNP, azide and cyanide have no effect on the resting or action potential of squid axons (Hodgkin and Keynes, 1955) but DNP and azide do depolarize the lobster axon (Senft, 1967). Segal (1968) found that the survival time of squid axons was decreased by DNP and cyanide. This decrease is not related solely to the cessation of active transport of sodium since axons in sodium-free medium also have a decreased

survival time. Cyanide and DNP depress the action potential in frog nerves without affecting the resting potential (Schoepflie and Block, 1959). These inhibitors (DNP, cyanide and azide) decrease ATP levels in lobster axons (Senft, 1957).

DNP, azide and cyanide release calcium from squid axons (Rojas and Hidalgo, 1968) and from frog nerves (Altura, 1968). The action of these agents in releasing calcium is not known but it is postulated that they are acting to inhibit the accumulation of calcium by intraaxonal sites (Rojas and Hidalgo, 1968). Blaustein and Hodgkin (1969) showed that calcium could be dialyzed from cyanide treated squid axoplasm 30 times faster than from normal axoplasm, indicating a release of calcium from axoplasmic sites. Studies by Carofli (1967) on rat liver showed that injection of DNP into rats decreased the amount of mitochondrial calcium and increased the calcium content of the microsomal fraction. The addition of DNP to Ca-45 labelled rat liver mitochondrial fractions caused the release of two thirds of the accumulated Ca-45 into the medium. These results indicate that DNP interferes with the accumulation and retention of calcium in liver mitochondria. DNP and other metabolic inhibitors may have the same effects on nerves.

Other agents which have been tested in the lobster axon are 1,5 difluoro 2,4 dinitrobenzene (FFDNB) and 2,4 dinitrofluorobenzene (FDNB). Both of these agents irreversibly abolish the action potential without affecting the resting potential of the lobster axon. These agents might be acting on membrane constituents which are responsible for conductance changes during excitation (Cooke, et al., 1968). Both FFDNB and FDNB form stable covalent bonds with proteins and therefore can change protein configuration or inactivate enzymes involved in nerve metabolism.

MATERIALS AND METHODS

I Dissection

All experiments were performed at room temperature (22–24°C) on isolated desheathed sciatic nerves of Rana pipiens. Frogs* were stored for periods up to six weeks in tap water at 5°C. Nerves were dissected free and placed in normal Ringer's solution. While being desheathed the nerves were pinned on a piece of cork and kept moist with normal Ringer's solution. The nerves were anchored to the cork with a piece of thread and all side branches were cut off. A small puncture was made in the sheath at the top of the nerve with a pair of jeweler's forceps. The sheath was picked up, teased away from the top of the nerve, and pulled from the nerve in much the same way as a sock is pulled off a foot. The entire procedure was carried out under a Bausch and Lomb dissecting microscope; the procedure lasted approximately 2 – 3 minutes.

II Solutions

All solutions were made using deionized water. Normal frog Ringer's solution contained: 110.8 mM NaCl, 2.0 mM KCl, 2.0 mM $NaHCO_3$, 0.1 mM $NaPO_4$, 1.8 mM $CaCl_2 \cdot H_2O$. Calcium-free Ringer's solution was made by eliminating the $CaCl_2$. Choline chloride Ringer's

* Frogs were obtained from Lemberger, Co., Wisconsin.

solution was prepared by substituting choline chloride for NaCl. Agents tested for their ability to antagonize spontaneous discharge were prepared by adding the desired quantity of the agent to calcium-free Ringer's solution. Solutions of 1,5 difluoro-2,4 dinitrobenzene (FFDNB) and other nitrobenzene derivatives were made by dissolving the compound in methanol and then adding this to calcium-free Ringer's solution. The final concentration of methanol was never more than 1.0 per cent. Control experiments were performed which showed that 1.0 per cent methanol had no measureable effect on the desheathed nerve. The pH of all solutions was kept at 7.2 unless otherwise specified. The pH was adjusted using HCl and NaOH.

III Electrical Measurements

Action potentials and spontaneous discharge of desheathed frog sciatic nerves were measured using standard electrophysiological techniques. The sciatic nerve is a compound nerve bundle; therefore the evoked action potential represents the summation of a number of action potentials moving at different velocities. As the distance from the stimulus increases the action potential travelling at different velocities separate so that the distance between the recording and the stimulating electrodes is important in determining the shape of the recorded spike. Recordings were made in air using stainless steel electrodes. The stimulating electrodes were 20 mm from the recording electrodes which were 15 mm apart. The amplitude of the spontaneous discharge and the elicited action potential were measured using a Tetronix Dual Beam Os-

cilloscope Type 502A. The recording electrodes were connected to the oscilloscope through a Grass Preamplifier Model DP98. A diagram of the experimental set up is shown in Fig. 1. Spontaneous discharge was measured directly from the oscilloscope screen by visually approximating the height of the spikes. This type of measurement takes into account both the number of axons discharging and the temporal summation of their discharge. The action potential was elicited with a stimulus of 0.1 msec duration and an amplitude which was supramaximal for A fibers (which have the highest threshold of stimulation). One or two pulses were applied to the nerve using a Grass Model S42 stimulator and the action potential height was read directly from the oscilloscope screen.

The experimental design for testing the action of different agents on spontaneous activity and the evoked spike involved first dissecting free a pair of nerves and placing them in normal frog Ringer's solution. Each nerve was then desheathed and tested separately. Immediately after desheathing the action potential and spontaneous activity of the nerve were recorded. The value of the action potential at this reading was equated to 100 per cent. All subsequent measurements were normalized to this value. This was done to compensate for the normal variation between nerves. The nerve was then placed in calcium-free Ringer's solution for 5 minutes after which time the action potential and spontaneous activity were again measured. The value of the spontaneous discharge at this time was designated as 100 per cent and all subsequent readings were expressed as a percentage of this value. As before this was done to minimize the inherent variation between nerves. The nerve

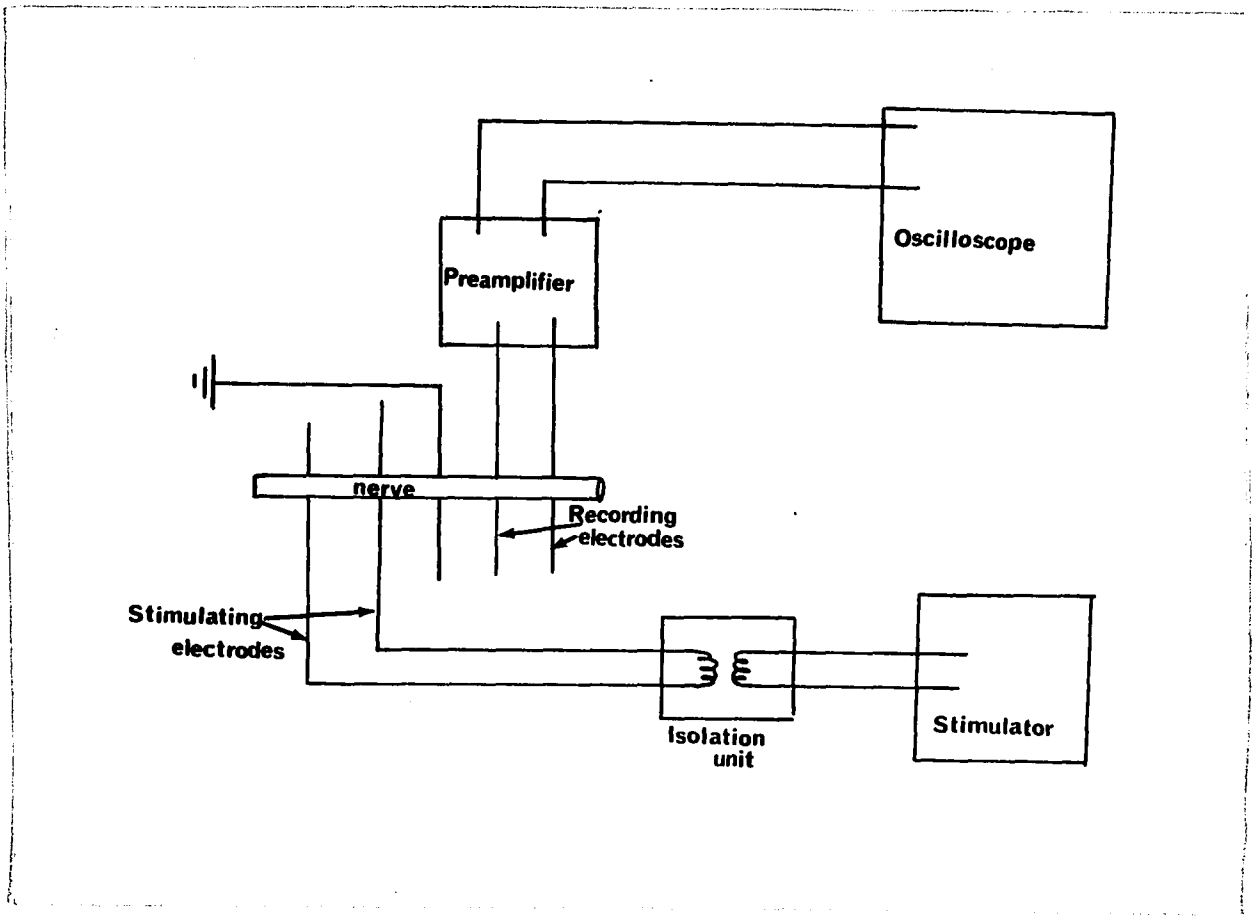

Fig. 1. A diagrammatic representation of the set up for recording both elicited action potentials and spontaneous discharge in the desheathed frog nerve.

was then placed in a test solution and the action potential and spontaneous discharge measured at various time intervals. The compounds to be tested for their ability to antagonize spontaneous activity were always added to calcium-free Ringer's solution.

IV Demarcation Potential Measurements

The demarcation potentials of desheathed frog sciatic nerves were measured in a lucite chamber containing three compartments (A, B, C) (Fig. 2). Compartments A and C have a capacity of 5 ml while compartment B has a capacity of 1 ml. The nerve was threaded through the pores in the barriers dividing the chamber into three compartments. The pores were then sealed with vaseline. Compartment B was filled with light mineral oil. Compartment A was filled with normal frog Ringer's solution while compartment C was filled with modified Ringer's solution. Potentials were measured with a high input impedance Bioelectric amplifier, Type NF1. The output of the amplifier was fed into a Tetronix oscilloscope, Model 502A. The bioelectric amplifier was connected to compartments A and C by silver: silver chloride electrodes which were inserted into 3 M KCl agar bridges. The resistance across the chamber was approximately 0.2 megohms.

Experiments were performed by putting normal frog Ringer's solution in both compartments A and C. A cotton wick moistened with Ringer's solution was then placed across the chamber connecting A and C and any potential difference (0-6 millivolts) was balanced out. The zero reading was checked in this way before each experimental reading. The

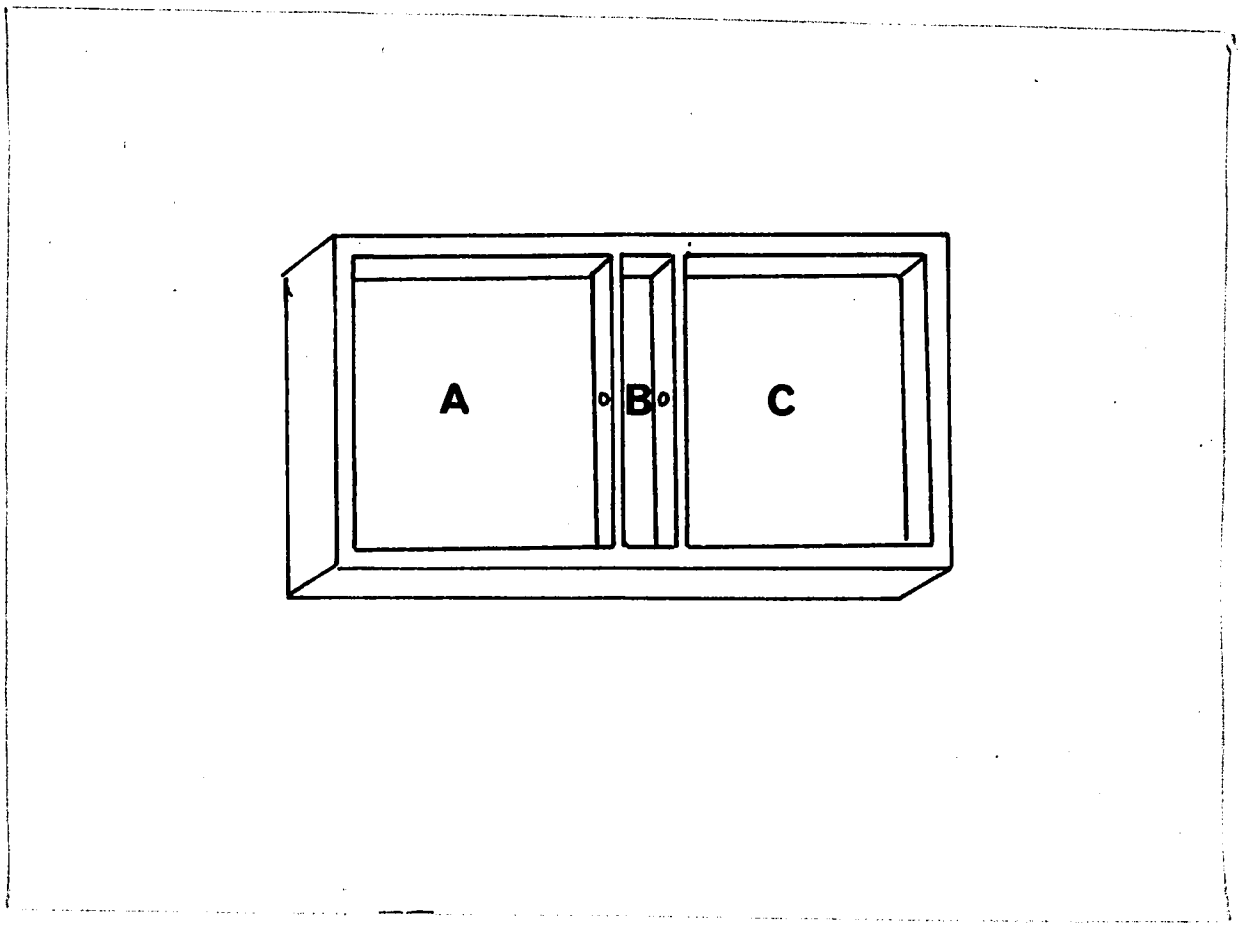

Fig.2. Lucite chamber used for demarcation potential measurements. Compartments A and C have the dimensions 3.0 cm x 3.8 cm x 0.5 cm. Compartment B has the dimensions 0.4 cm x 3.8 cm x 0.5 cm.

potential of the nerve with normal Ringer's solution on both sides was determined after 5 minutes equilibration. Only nerves with a potential difference of less than 0.6 millivolts were used. The normal Ringer's solution in compartment C was replaced with calcium-free Ringer's solution and the potential change was recorded over a 5 minute period. After the nerve was depolarized by calcium-free Ringer's solution the test solution was added to side C and any subsequent change was recorded. The test solution at the end of an experiment was always replaced by normal Ringer's solution to see if the resting potential returned to the original value.

V Calcium-45 Efflux Studies

Calcium-45 efflux studies were performed on paired nerves. One half of each pair served as the control. Radioactive calcium* was made into a stock solution with a final concentration of 10 uc/ml of Ca-45 in normal Ringer's solution. Frog sciatic nerves were removed and soaked in the stock solution for 2 hours at room temperature. They were then desheathed in normal Ringer's nonradioactive solution and the washout study was begun. Each nerve was placed in the barrel of a 5 ml syringe which contained 3 ml of Ringer's solution (the composition varied with the experiment). The Ringer's solution was replaced every 10 minutes with fresh solution throughout the duration of the experiment. A 1 ml aliquot was removed from each 3 ml sample and placed in a polyethylene scintillation vial. At different times during the washout test agents were added

* Calcium-45 was obtained from New England Nuclear Corp., Mass.

to the Ringer's solution. At the end of each experiment the tissues were blotted, weighed and ashed at 600°C. The ashed nerves were dissolved in 3 ml of 0.1 N HCl and a 1 ml aliquot was removed. Each sample was placed in a counting vial and 10 ml of scintillation fluor was added. Each 10 ml collection sample and the acid dissolved ashed nerves was counted in a liquid scintillation spectrometer. The efficiences and the quench correction curves were determined by the channel ratio method. All counts were corrected for radioactive decay and the background count was automatically subtracted.

The scintillation cocktail contained the following reagents: 6 liters of dioxane (spectroquality), 1.8 g 1,4 Bis-2 (4 methyl-5 phenyl oxazoyl) benzene, 180 g naphthalene, 36 g 2,5 - diphenyloxazole, and 600 ml of ethoxy ethanol.

The data obtained from calcium-45 washout studies was plotted in two ways. Desaturation curves which describe the loss of tissue radioactivity as a function of time were made. The loss of tissue radioactivity was expressed as a per cent of the initial radioactivity in these curves. Rate coefficient curves which give a more sensitive indication of the change in the rate of efflux were also constructed. This type of curve accounts for the amount of radioactivity remaining in the nerve during the collection period and expresses the efflux during this period as a percentage of this quantity.

VI Adenosine 5' Triphosphate Measurements

Adenosine 5' triphosphate (ATP) was determined by fluorimetry

using the method of Greengard (Bergmeyer, 1963). This method is based on two principles: 1) that the conversion of triphosphopyridine nucleotide (TPN) to reduced triphosphopyridine nucleotide (TPNH) is quantitatively related to the concentration of ATP and 2) that TPNH fluoresces while TPN does not. The reactions involved are shown in Fig. 3.

The measurements were made using a Farrand filter fluorometer, Model A2. The primary glass filter used was a Corning 7-37 (365 mu) filter and the secondary filters were Corning 4-70 and 3-73 (460 mu) filters. The enzymes* used were dissolved in cold deionized water. Solutions were made to give a final concentration of 0.08 Iu/ml for each enzyme in the test solution.

Paired nerves were dissected free and desheathed. One of each pair was soaked in the test Ringer's solution and the other half in the control solution. At the end of the soaking period the nerves were blotted, weighed and plunged into test tubes containing 1.5 ml of 0.1 M triethanolamine buffer which was heated to 100°C in a boiling water bath. The nerves were kept at this temperature for 40 seconds after which they were placed in an ice bath. The contents of each tube was then homogenized and 2 ml of chloroform (spectrometric quality) was added to the homogenate. The tube contents were then mixed for 3 minutes and centrifuged for 10 minutes at 3,000 G. A 0.3 ml aliquot of the supernatant was used for the ATP measurements.

Each sample to be tested contained: 0.3 ml aliquot of nerve

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* Glucose 6-phosphate dehydrogenase (yeast) and hexokinase (yeast) were obtained from either Sigma Co., Mo. or Boehringer Co. (Cal. Biochem. distributors).

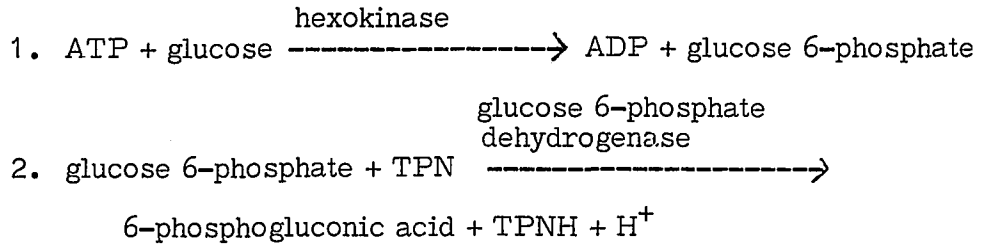

Fig. 3. Two enzymatic reactions involved in the assay for adenosine 5' triphosphate.

extract or an ATP standard, 0.125 ml of 0.1 M triethanolamine buffer, 0.15 ml of reaction mixture and enough deionized water to bring the final concentration in each tube to 0.9 ml. The reaction mixture contained: 2 ml of TPN (5×10^{-5}M), 0.2 ml of 1.0 M glucose, 0.2 ml of 0.15 M $MgCl_2$ and 0.6 ml of 0.02 M EDTA. The triethanolamine fibber was made by dissolving 1.857 g of triethanolamine hydrochloride in a little distilled water and bringing the pH up to 8.0 with 63 ml of 0.1 N NaOH. The final volume of buffer was then brought up to 100 ml.

After the background fluorescence of each sample tube was measured, 0.05 ml of hexokinase and 0.05 ml of glucose 6-phosphate dehydrogenase was added to each tube. The reaction was completed in about 25 minutes and the fluorescence of each tube was measured. The fluorescence values for the ATP standards were plotted against the known amount of ATP in each standard. The amount of ATP in each sample tube was then determined by using the standard curve. All results were expressed as umoles of ATP /gram wet weight of nerve.

RESULTS

I Divalent Cations

A. Effect on Spontaneous Discharge and Action Potential

Immersed in a calcium deficient Ringer's solution, the desheathed nerve develops spontaneous repetitive discharge within 2 minutes. A typical recording of spontaneous discharge in a nerve after 5 minutes in calcium-free Ringer's solution is shown in Figure 4. The amplitude of the spontaneous discharge reaches a maximum (100-300 volts) after 4 minutes and then decreases gradually over the next 40 minutes (Fig. 5). The action potential amplitude rapidly decreases in calcium-free Ringers solution to about 40 per cent of the initial value (15-25 mvolts) and then stabilizes at this point during the next 40 minutes. Recordings of nerve action potentials before and after soaking in calcium-free Ringer's solution for 5 minutes are shown in Figure 6. The action potential amplitude of nerves soaked in calcium-free Ringer's solution over this time period was approximately 40% lower than in nerves soaked in normal Ringer's solution. If calcium (>0.25mM) was added back to the medium nerves which had been spontaneously firing stopped immediately and the action potential amplitude increased from 40 to 60 per cent of the initial value recorded immediately after desheathing. Table 1 gives the average values for both the action potential and spontaneous discharge amplitude recorded from desheathed nerves in calcium-free Ringer's solution.

A number of divalent cations were tested for their ability to

antagonize spontaneous discharge induced by lack of calcium. The action of barium, strontium and magnesium was examined and barium was the only alkali earth metal which did not completely antagonize spontaneous discharge at a concentration of 1.8 mM (the concentration of calcium in normal Ringer's solution). $ZnCl_2$, $CoCl_2$, $NiCl_2$ and $MnCl_2$ (1.8 mM) were also tested and found to antagonize spontaneous activity. Figure 7 shows the effect of placing a nerve in a solution containing 0.45 mM of each divalent cation for 5 minutes. These nerves were pretreated in calcium-free Ringer's solution for 5 minutes and were spontaneously firing when placed in the solution containing the divalent cation. The initial value of the action potential after desheathing was equated to 100 per cent. The value of spontaneous discharge after 5 minutes in calcium-free solution was equated to 100 per cent. The bars which are shaded represent the per cent spontaneous discharge remaining after 5 minutes in the test solution while the white bars represent the per cent amplitude of the evoked spike remaining after 5 minutes in the test solution. From the data collected using different concentrations of divalent cations, dose response curves were calculated (Fig. 8). A least squares regression analysis for all the lines indicated that their slopes were not significantly different. From this data the relative efficiences of each cation to antagonize spontaneous discharge was calculated to be: Ca (1 ± 0.4), Mn (1.24 ± 0.1), Co (1.69 ± 0.05), Ni (2.9 ± 0.22), Zn (4.1 ± 0.1), Mg (5.3 ± 0.07), Sr (5.3 ± 0.32). These values were obtained by taking the concentration of calcium which decreased spontaneous discharge by 50 per cent and equating that value to 1.0. The concentrations of the other cations which

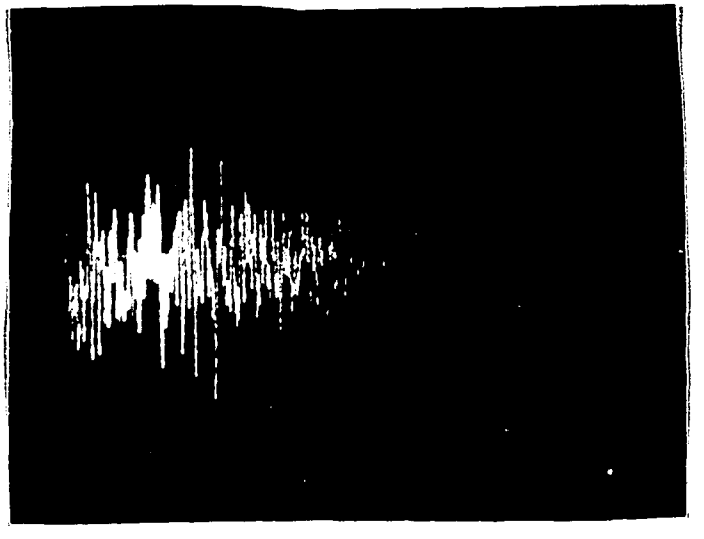

Figure 4. Spontaneous discharge in a desheathed frog sciatic nerve recorded after 5 minutes in calcium-free Ringer's solution. (Sweep speed = 5 milliseconds/centimeter; sensitivity = 100 microvolts/centimeter).

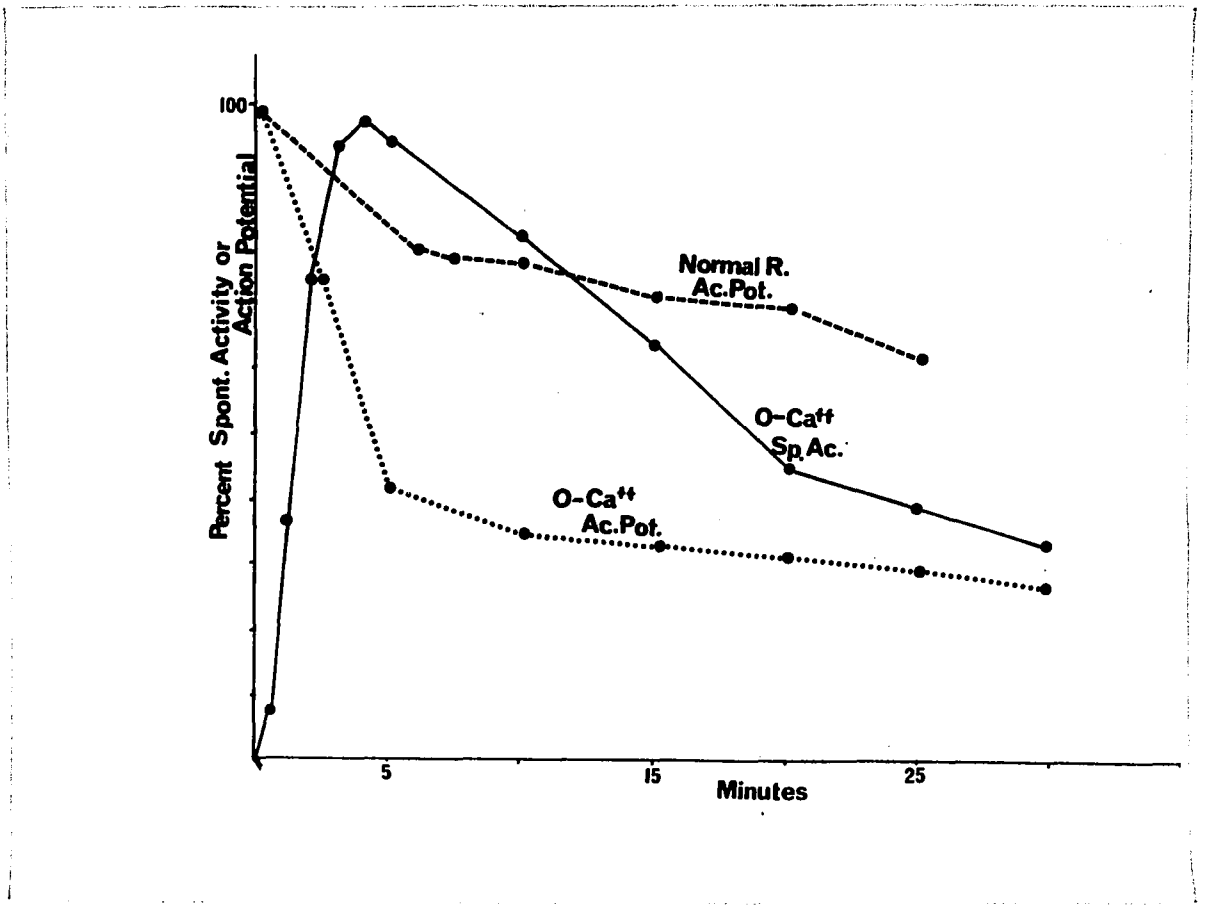

Fig. 5. Time course of spontaneous activity (solid line) and the action potential amplitude (dotted line) of desheathed frog sciatic nerves in calcium-free Ringer's solution (0-Ca^{++}). The action potential amplitude (dashed line) in normal Ringer's solution is also shown. Each point is the mean of 12 separate experiments (12n). The standard error ($\pm$ SE) is given for each point.

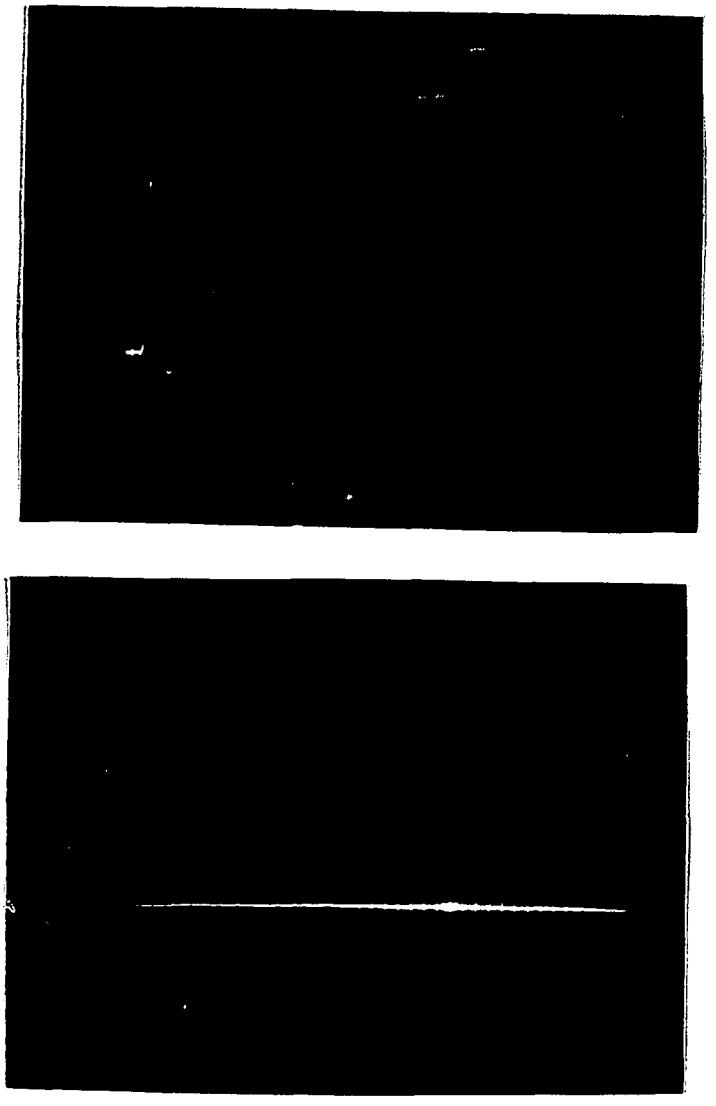

Figure 6. Top: Action potential recorded from a desheathed frog sciatic nerve immediately after desheathing.
Bottom: Action potential recorded from a desheathed nerve after 5 minutes in calcium-free Ringer's solution. (Sweep speed = 5 milliseconds/centimeter; sensitivity = 5 millivolts/centimeter).

Table 1. The effect of the length of exposure to calcium-free solution on the action potential and spontaneous discharge amplitudes in desheathed frog nerves. Each value is an average of 6 nerves. The standard error is given for each value.

Time (minutes)	Action Potential (millivolts)	Spontaneous Discharge (microvolts)
0	22 ± 1.5	0
0.5	21 ± 1.6	20 ± 10
1.0	17 ± 1.5	70 ± 13
2.0	12 ± 1.9	160 ± 18
3.0	11 ± 1.4	200 ± 15
5.0	8 ± 1.4	200 ± 11
10.0	8 ± 1.4	148 ± 19
20.0	7 ± 1.0	118 ± 12
30.0	7 ± 0.9	100 ± 14
60.0	6 ± 0.7	80 ± 11

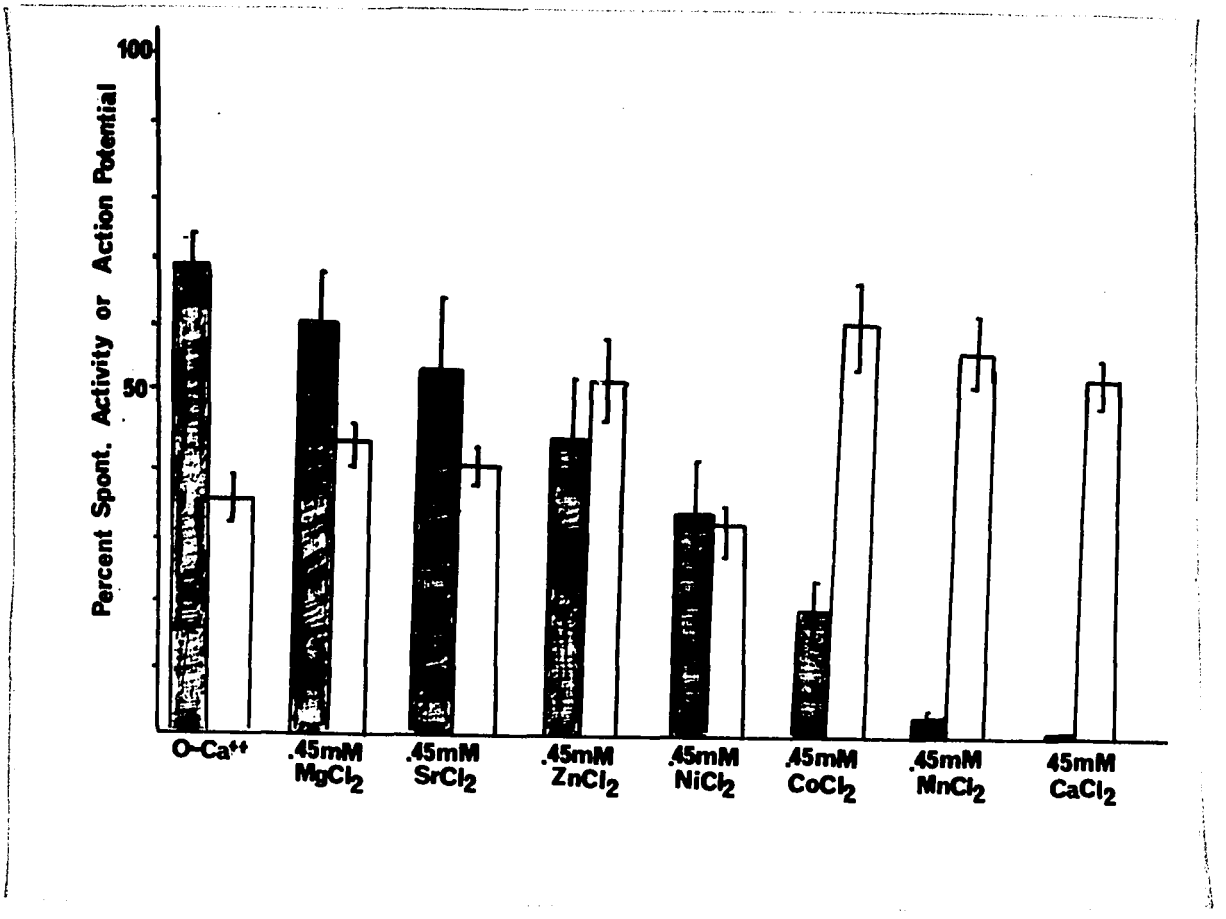

Fig. 7. The effects of divalent cations on the spontaneous discharge and action potential amplitude of calcium deficient nerves. The shaded bars represent the per cent spontaneous discharge remaining after 5 minutes treatment in the test solution. The white bars represent the per cent action potential amplitude remaining after 5 minutes in the test solution. Each bar is a mean of 6 separate experiments. The standard error (±SE) is shown for each bar.

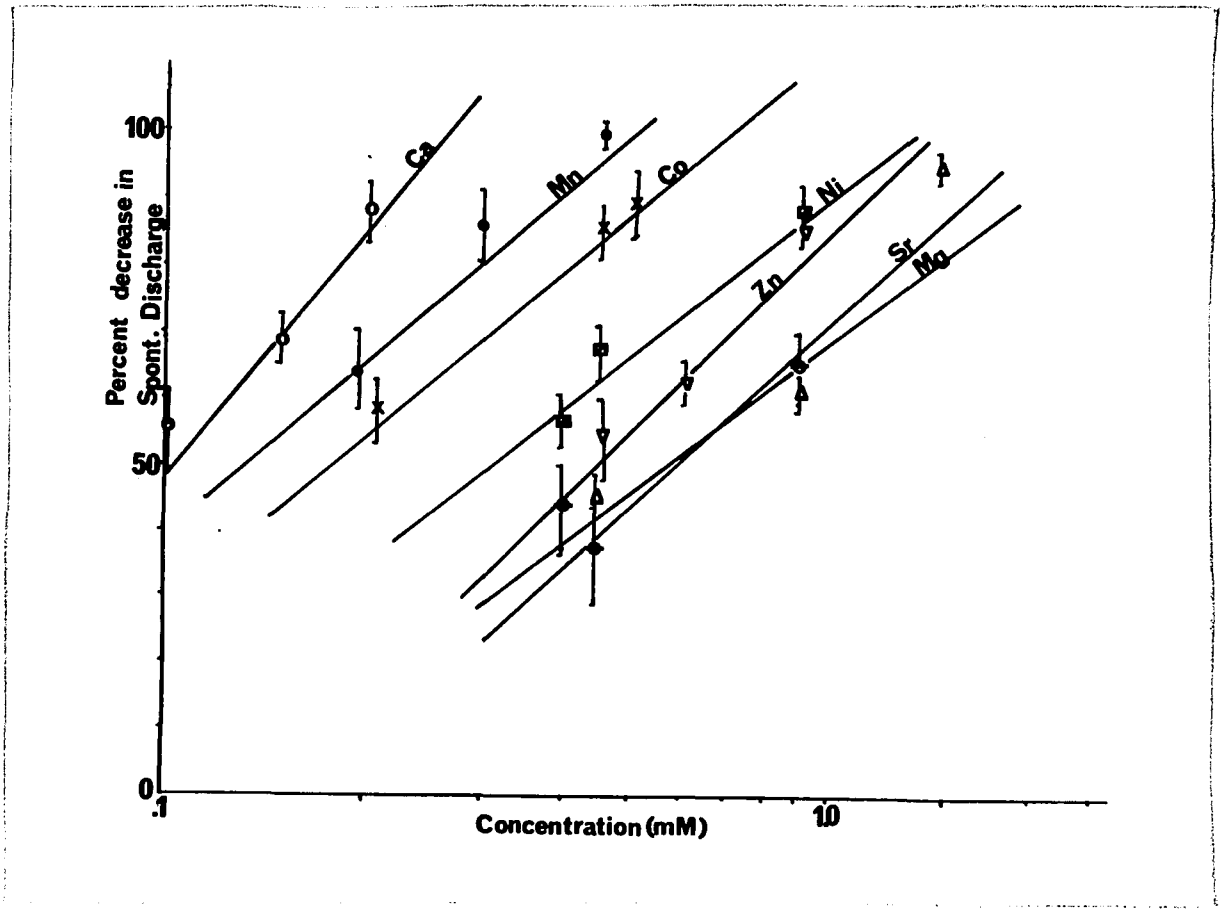

Fig. 8. Concentration-response regression lines for antagonism of spontaneous discharge by 7 divalent cations. The per cent decrease in spontaneous discharge was determined for each concentration of cation. Each point is a mean of at least 6 separate experiments. The standard error (±SE) is shown for each point.

Values for standard errors of the slopes

$MnCl_2$	.86	± .10
$NiCl_2$	.804	± .22
$CaCl_2$	1.34	± .44
$MgCl_2$	.76	± .07
$CoCl_2$	.82	± .15
$SrCl_2$	.95	± .32
$ZnCl_2$	1.09	± .10

decreased spontaneous discharge by 50 per cent were then expressed in terms of calcium.

B. Demarcation Potential Measurements

Studies were done to investigate whether the cations which antagonize spontaneous activity also reversed the depolarization caused by calcium deficiency. Experiments were carried out in which nerves were first equilibrated with normal Ringer's solution and then calcium-free Ringer's solution was added to chamber C. The demarcation potential was measured at 2, 5, 10 and 15 minute intervals. The nerves reached their maximum value of depolarization -0.75 ± 0.01 mv within 5 minutes and maintained this depolarization for the next 10 minutes (Fig. 9). If 2 mM EGTA* was added to the calcium-free Ringer's solution in compartment C the depolarization after 5 minutes reached a value of -1.2 ± 0.08 mv. Experiments were performed in calcium-free Ringer's solution containing $MnCl_2$, $MgCl_2$ and $BaCl_2$ (1.8 mM). In these experiments chamber C contained calcium-free Ringer's solution for 5 minutes after which time the solution containing the divalent cation was added. It was found that 1.8 mM $MnCl_2$ reversed the depolarization caused by calcium-free Ringer's solution within 1 minute (Fig. 9). The solution containing 1.8 mM $MgCl_2$ was much slower at reversing the depolarization caused by calcium deficiency, and at the end of 5 minutes in $MgCl_2$ solution the nerve was still slightly depolarized (-0.25 ± 0.03 mv). The solution containing 1.8 mM $BaCl_2$ caused no repolarization. In fact, after 5 minutes in the

* EGTA-ethylene glycol bis (amino ethylether) N,N-tetraacetic acid

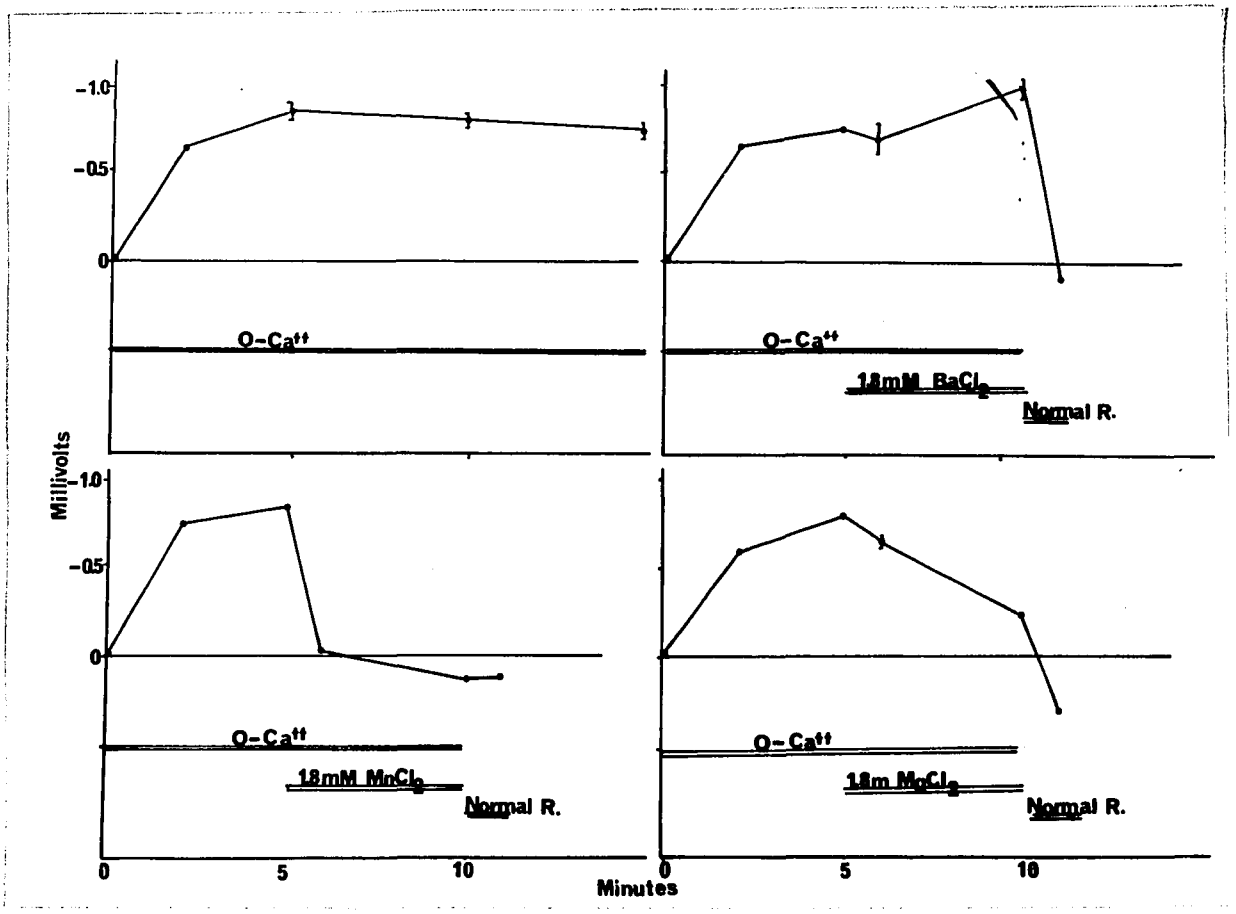

Fig. 9. The effect of calcium-free Ringer's solution in the presence and absence of other divalent cations on the demarcation potential in the desheathed frog nerve. The bars underneath the graphs indicate the solutions bathing the nerves at any given time. Each point is a mean of 6 separate experiments. The standard error (±SE) is given for each point.

$BaCl_2$ solution the depolarization was slightly increased (-1.0 ± 0.02).

Experiments were also performed using varying concentrations of potassium in the bathing medium in order to determine the magnitude of the transmembrane change. Figure 10 shows the relationship between the external potassium concentration and the change in demarcation potential. Huxley and Stampfli (1951) recorded resting potentials from single myelinated nerve fibers using an external current to compensate for the injury current between the two nodes (one node was placed in isotonic KCl while the other was kept in normal Ringer's solution). From this and other measurements it can be concluded that the demarcation potential changes measured in this chamber are approximately one third to one quarter of the actual membrane potential changes.

C. Calcium-45 Efflux Studies

Experiments measuring calcium-45 efflux were carried out in order to correlate the ability of cations to release calcium with their ability to antagonize spontaneous discharge. Divalent cations release calcium from the frog nerve in the following order: Ca, Sr>Ba>Mg, Zn, Ni>Mn (B. Altura, 1968). Experiments were done on the desheathed nerve which gave similar results. Nerves were washed out in normal Ringer's solution for the first 30 minutes and then in calcium-free Ringer's solution for the next 10 minutes. Following this procedure they were washed out in Ringer's solution containing 1.8 mM $SrCl_2$, $CaCl_2$ or no divalent cation at all (0-Ca). When nerves were washed out in calcium-free solution for the last 40 minutes of the efflux experiment slightly

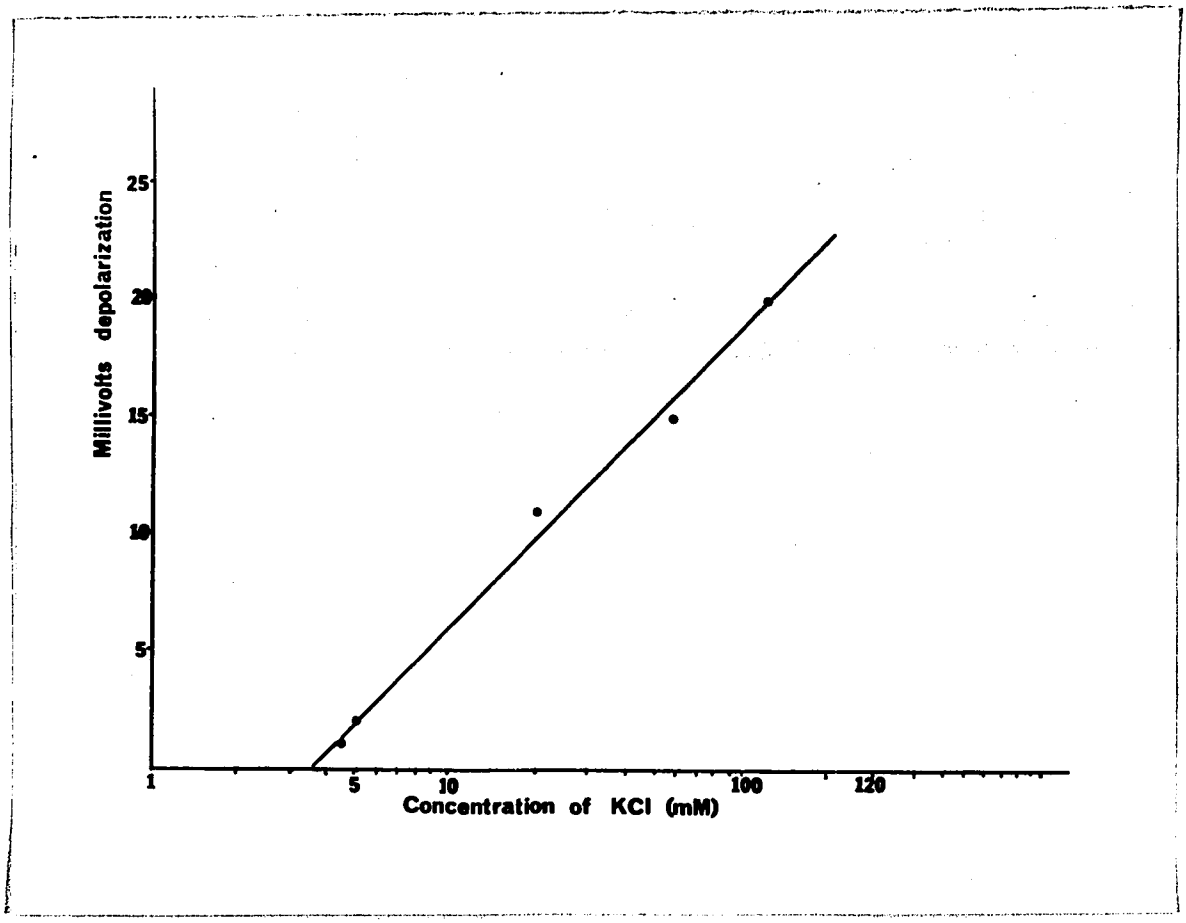

Fig. 10. The effect of changes in external KCl concentration on the demarcation potential measurements in the frog nerve. Each point is a mean of 4 separate experiments.

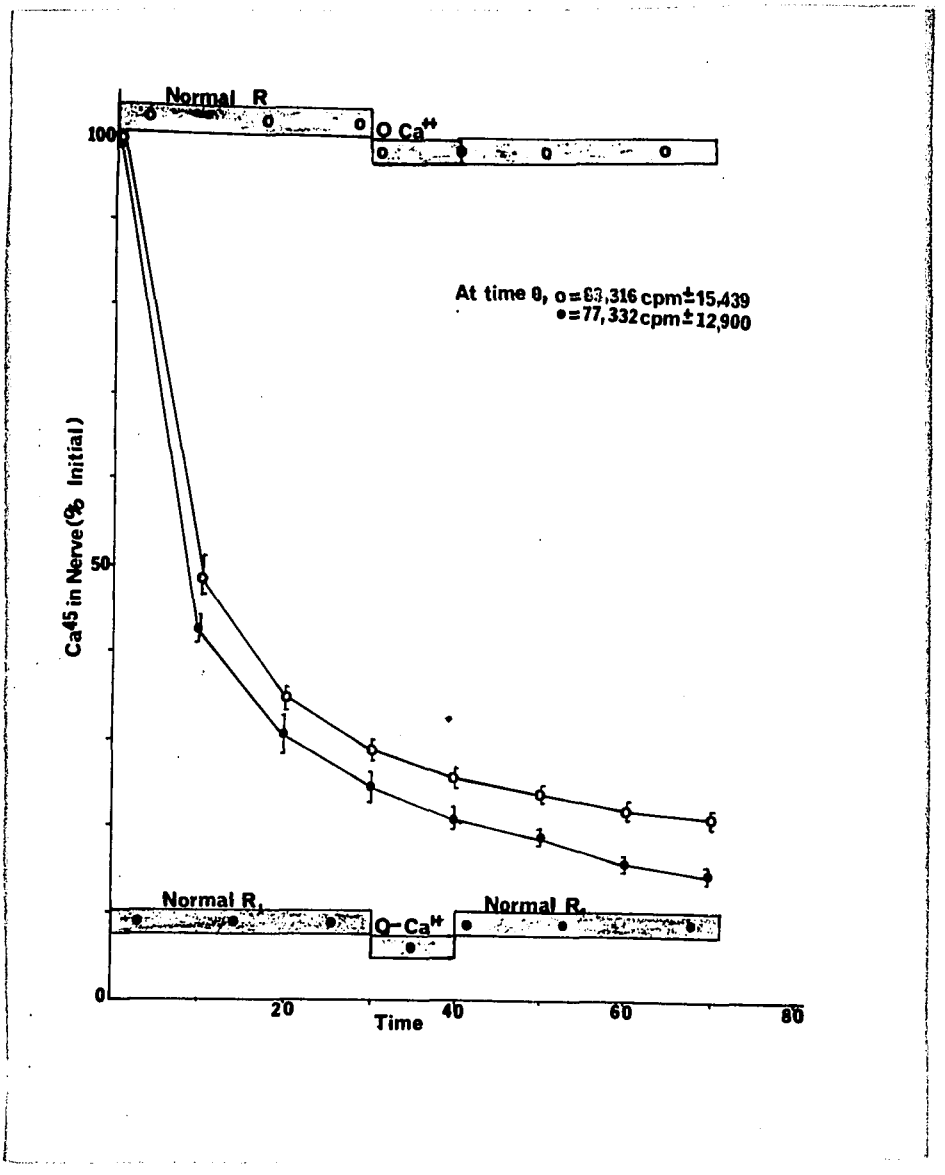

Fig. 11. The effect of calcium-free Ringer's solution on the percent of Ca-45 remaining in isolated paired desheathed nerves. The experimental nerves were washed out in normal Ringer's solution for the first 30 minutes at which time calcium-free Ringer's solution was added for the remainder of the experiment. The control nerves were washed out in normal Ringer's solution for the entire experiment. Each point is a mean of 3 nerves (3n). The standard error (±SE) is given for the latter portion of the curve. Notations at the top and bottom of the graph refer to the open and closed circles respectively.

more Ca-45 remained in the nerves ($P<0.01$) than in the control nerves which were kept in normal Ringer's solution (Fig. 11). Nerves which were washed out with 1.8 mM $MnCl_2$ Ringer's solution behaved in a way similar to those in calcium-free Ringer's solution ($P<0.01$) (Fig. 12). On the other hand, nerves washed out in 1.8 mM $SrCl_2$ Ringer's solution for the last 30 minutes behaved in the same manner as those in normal Ringer's solution, that is, there was not a significant difference between the Ca-45 remaining in the two sets of nerves ($P>0.05$) (Fig. 13).

A series of experiments was also done to determine whether good calcium releasers (such as strontium and barium) were releasing the same fraction of calcium that calcium itself released - i.e., the self exchangeable fraction. Nerves were washed out in calcium-free Ringer's solution for 30 minutes. $BaCl_2$ Ringer's solution was then added for 20 minutes after which normal Ringer's solution was introduced for the rest of the experiment. The results, plotted as rate coefficient versus time, (Fig. 14) indicate that barium does release the same fraction of calcium as calcium itself. The calcium efflux from nerves in normal Ringer's solution which had previously been exposed to barium was smaller than the control set which had been exposed only to calcium-free Ringer's solution for the entire 50 minutes before the normal Ringer's solution was added.

A similar experiment investigated the possibility that the fraction of calcium that the poor calcium releasers such as nickel were releasing was different from the fraction of calcium that the potent calcium releasers such as barium were releasing. The experiments showed that when $BaCl_2$

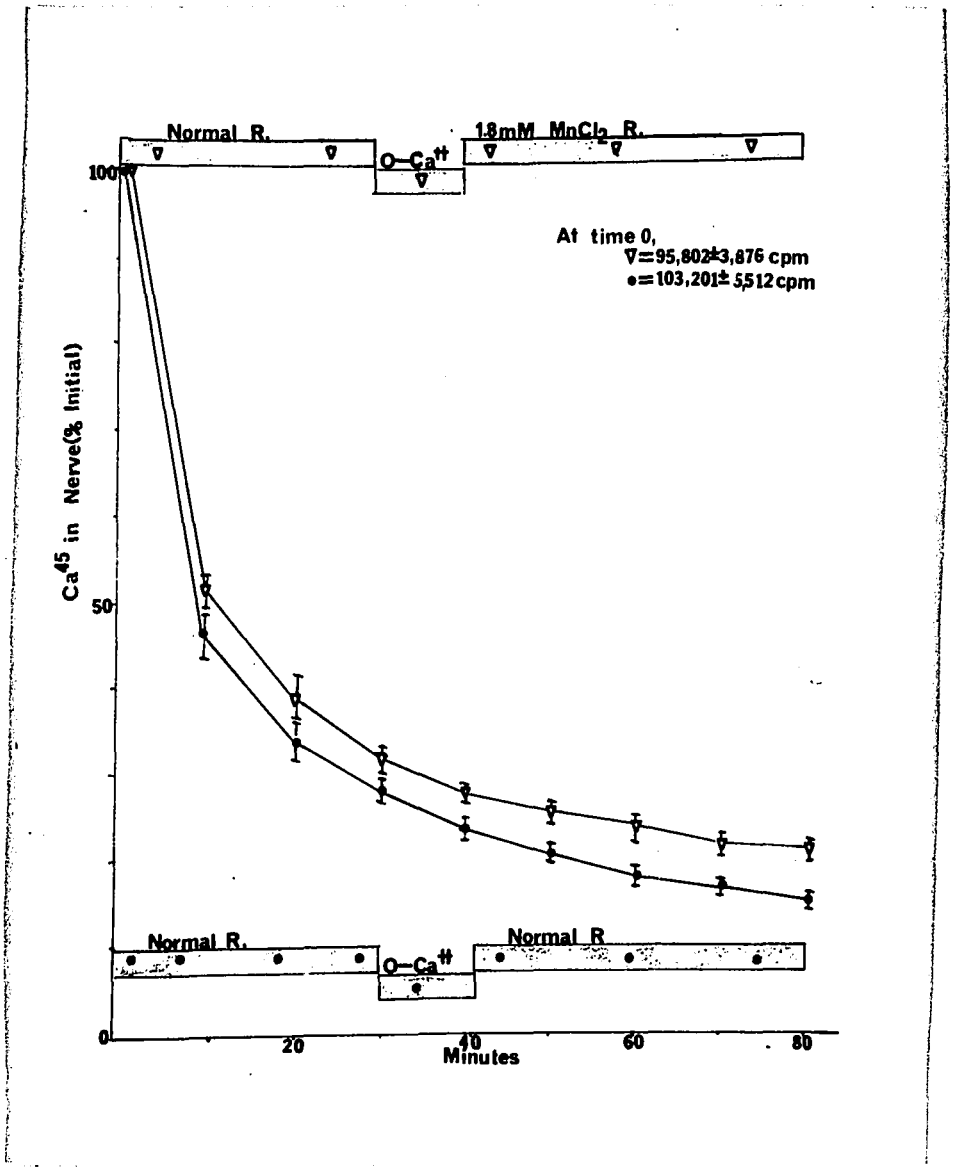

Fig. 12. The effect of 1.8 mM $MnCl_2$ Ringer's solution on the percent of Ca-45 remaining in isolated paired desheathed frog nerves. The experimental nerves were washed out in normal Ringer's solution for the first 30 minutes, then calcium-free Ringer's solution was added for 10 minutes after which 1.8 mM $MnCl_2$ Ringer's solution was added. The control nerves were washed out in normal Ringer's solution for 30 minutes, then calcium-free Ringer's solution was added for 10 minutes and normal Ringer's solution was added for the remainder of the experiment. Each point is a mean of 6 separate experiments (6n). The standard error ($\pm$SE) is given for the latter portion of the curve. Notations at the top and bottom of the graph refer to the open and closed circles respectively.

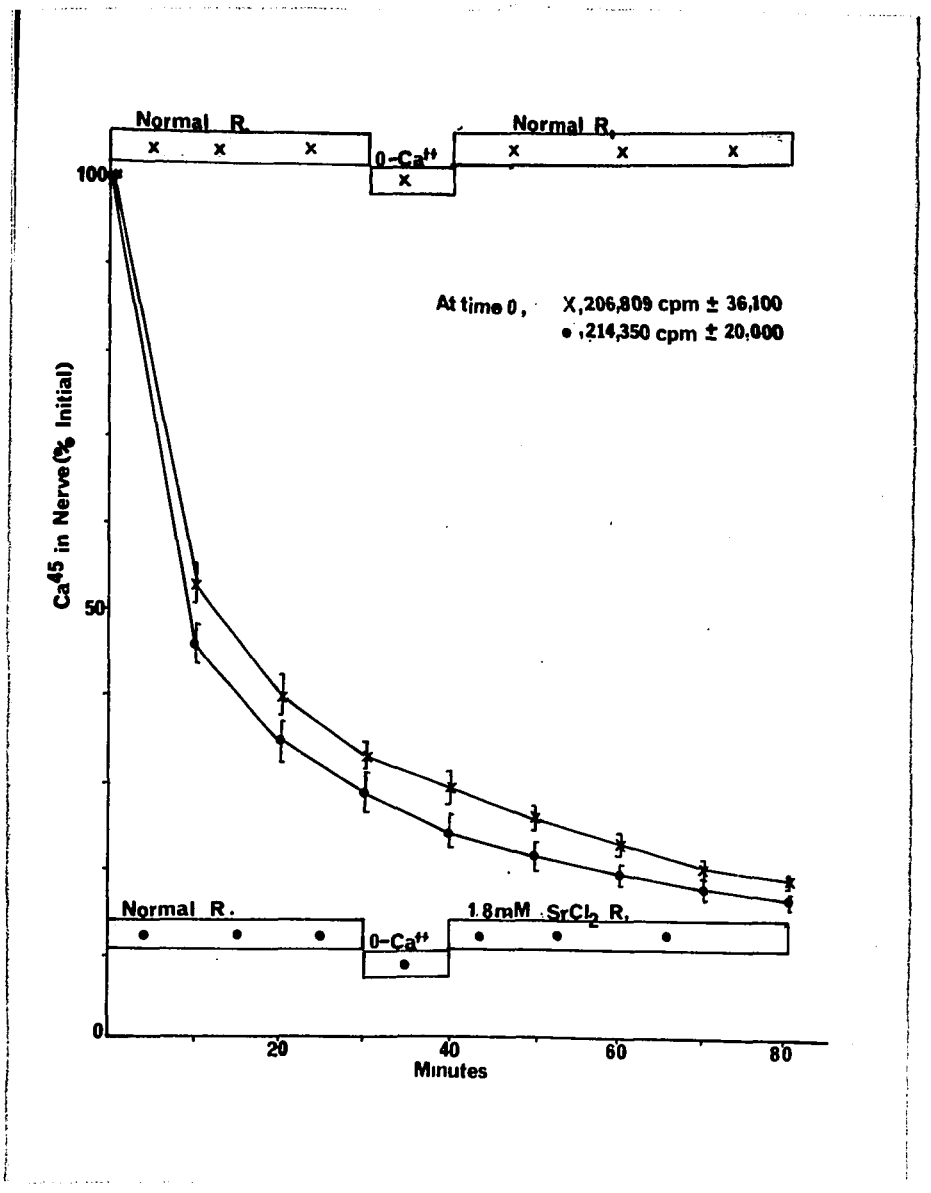

Fig. 13. The effect of 1.8 mM $SrCl_2$ Ringer's solution on the percent of Ca-45 remaining in isolated paired desheathed nerves. The experimental nerves were washed out in normal Ringer's solution for the first 30 minutes then calcium-free Ringer's solution was added for 10 minutes after which 1.8 mM $SrCl_2$ Ringer's solution was added. The control nerves were washed out in normal Ringer's solution for 30 minutes, then calcium-free Ringer's solution was added for 10 minutes after which normal Ringer's solution was put back. Each point is a mean of 3 separate experiments (3n). Notations at the top and bottom of the graph refer to the open and closed circles respectively.

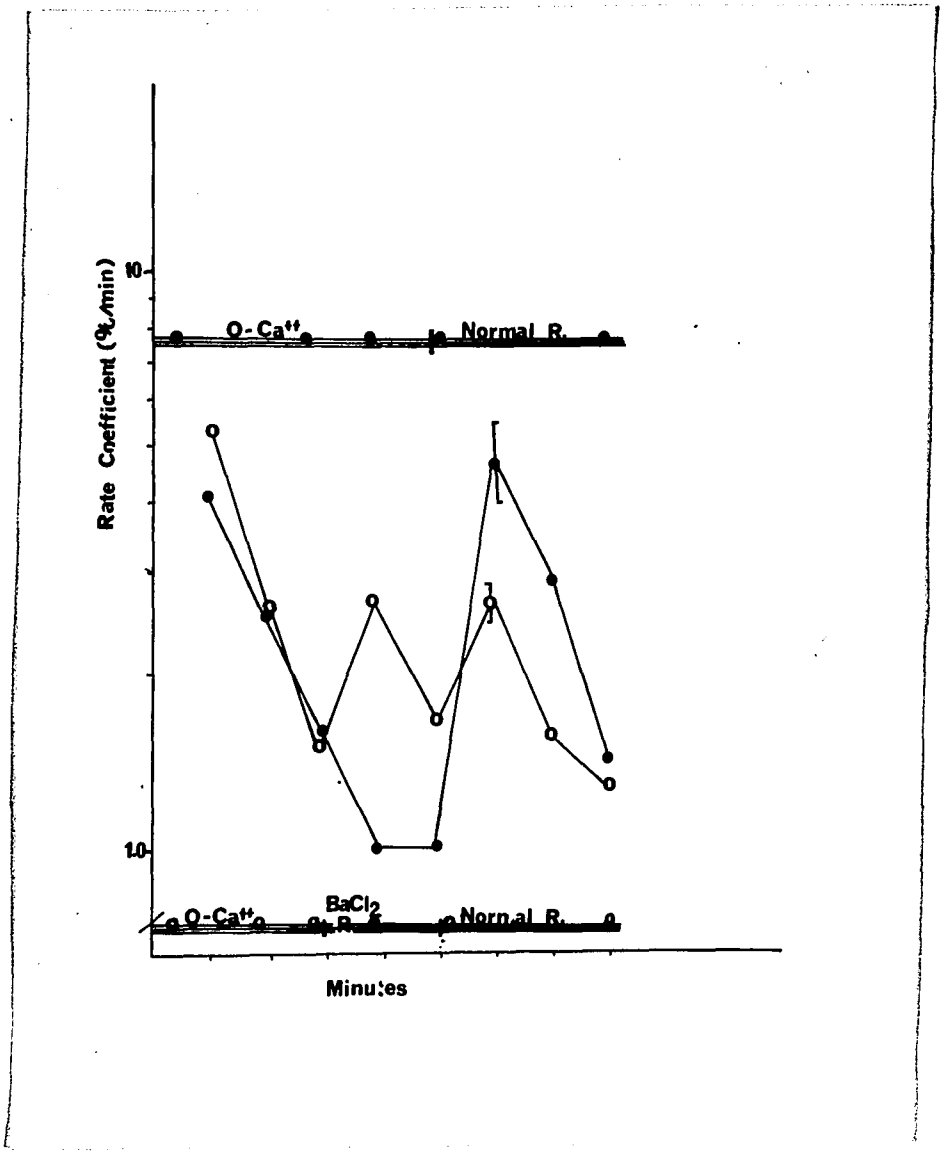

Fig. 14. The effect of 1.8 mM $BaCl_2$ Ringer's solution on the subsequent release of Ca-45 by normal Ringer's solution in isolated paired desheathed nerves. The experimental nerves were washed out in calcium-free Ringer's solution for 30 minutes, then 1.8 mM $BaCl_2$ was added for 20 minutes after which normal Ringer's solution was added for the remainder of the washout. Control nerves were soaked in calcium-free Ringer's solution for the first 50 minutes after which normal Ringer's solution was added for the remainder of the washout. Each point is a mean of 3 separate experiments (3n). The standard error ($\pm$SE) is given for 2 points. Notations at the top and bottom of the graph refer to the open and closed circles respectively.

Ringer's solution was added to the washout medium before $NiCl_2$ Ringer's solution the efflux that was usually caused by nickel was eliminated (Fig. 15). The calcium that nickel was releasing was therefore indistinguishable from the calcium that barium released.

D. ATP Measurements

The effect of calcium deficiency on the ATP content of the nerve was also investigated. Pairs of nerves were desheathed and one of each pair was placed in a normal Ringer's solution for 25 minutes while the other half was placed in a calcium-free Ringer's solution. It was found that (Table 2a) there was a significant decrease in the ATP content in nerves that had been exposed to calcium-free Ringer's solution for 25 minutes. An experiment was then performed to investigate the possibility that any agent which depolarized the nerve would cause a decrease in the ATP content. Nerves were soaked in 3 mM KCl normal Ringer's solution for 25 minutes and compared to controls which had been soaked in normal Ringer's solution (Table 2e). This concentration of KCl causes a depolarization of approximately the same magnitude as that caused by calcium-free Ringer's solution. There was no statistical difference between the two groups of nerves.

A series of experiments was done using (1.8 mM) $MnCl_2$, $SrCl_2$ and $CoCl_2$ Ringer's solution to ascertain if these cations could prevent the decrease in ATP content caused by calcium deficiency. No differences were found between nerves soaked in solutions containing manganese or cobalt and those containing no divalent cation (Table 2b, d). Nerves

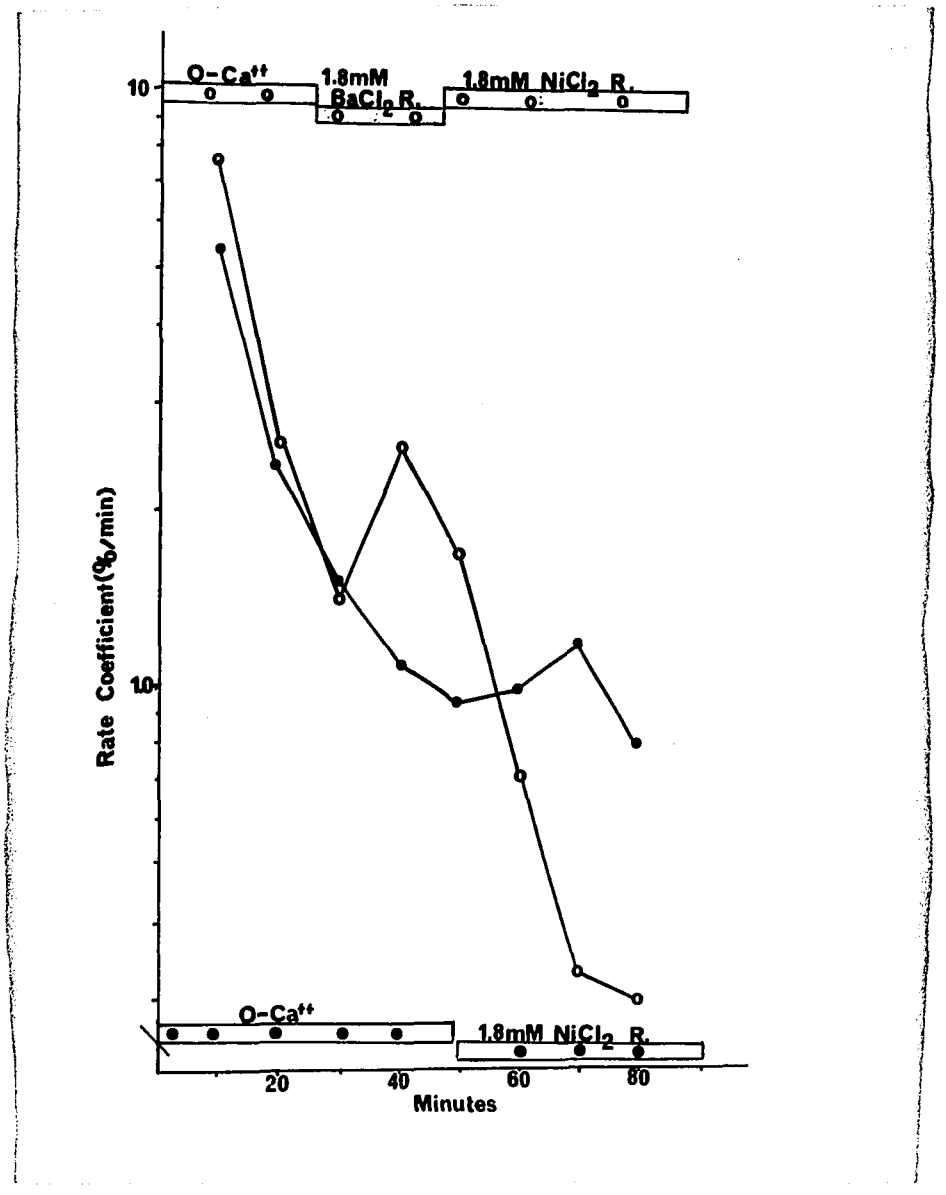

Fig. 15. The effect of 1.8 mM $BaCl_2$ Ringer's solution on the subsequent release of Ca-45 by 1.8 mM $NiCl_2$ Ringer's solution in isolated paired desheathed nerves. The experimental nerves were washed out in calcium-free Ringer's solution for 30 minutes, then 1.8 mM $BaCl_2$ Ringer's solution was added for 20 minutes, after which 1.8 mM $NiCl_2$ Ringer's solution was added for the remainder of the experiment. Control nerves were washed out in calcium-free Ringer's solution for the first 50 minutes, then 1.8 mM $NiCl_2$ Ringer's solution was added. Each point is a mean of 3 experiments (3n). Notations at the top and the bottom of the graph refer to the open and closed circles respectively.

Table 2. Effect of cations on the ATP content of desheathed frog serves soaked in Ringer's solution for 25 minutes.

Exp. set	# pairs of nerves tested	Ringer's solution	uM ATP /gm wet wt.	Significance
a	12	$0\text{-}Ca^{++}$	0.625 ± 0.05	$P < 0.0005$
		1.8 mM $CaCl_2$ (normal R.)	0.785 ± 0.11	
b	6	$0\text{-}Ca^{++}$	0.942 ± 0.06	$P \leq 0.10$
		1.8mM $CoCl_2$- $0\text{-}Ca^{++}$	0.978 ± 0.07	
c	7	$0\text{-}Ca^{++}$	0.9852 ± 0.05	$P < 0.005$
		1.8 mM $SrCl_2$- $0\text{-}Ca^{++}$	0.8544 ± 0.02	
d	5	$0\text{-}Ca^{++}$	0.962 ± 0.1	$P < 0.20$
		1.8 mM $MnCl_2$- $0\text{-}Ca^{++}$	0.868 ± 0.08	
e	5	1.8 mM $CaCl_2$- (normal R.)	0.7877 ± 0.15	$P < 0.25$
		1.8 mM $CaCl_2$+ 3 mM KCl	0.722 ± 0.17	

which were soaked in $SrCl_2$ Ringer's solution showed a significant decrease in ATP content as compared with control nerves soaked in calcium-free Ringer's solution (Table 2c).

II The Effect of Variations in Hydrogen Ion Concentration

A. Spontaneous Discharge and Action Potential Amplitude

The effect of changes in hydrogen ion concentration on both spontaneous activity and the action potential amplitude was studied. Nerves were placed in calcium-free Ringer's solution whose pH varied from 4.6 to 9.9. Both spontaneous activity and the action potential amplitude were measured every 5 minutes. Figure 14 summarizes the effect of changes in pH on both the spontaneous activity and evoked potentials after 25 minutes in the test solution. At pH 9.9 both spontaneous activity and the evoked spike were reduced after 25 minutes, while at pH 5.5 spontaneous discharge was significantly reduced with little or no effect on the action potential. At pH 4.6 spontaneous discharge was completely abolished and the evoked spike was only slightly modified. The optimum pH range for spontaneous discharge is from 6.0 to 8.0 while that for the action potential is broader. The pH of nerves soaked in normal Ringer's solution was varied from 5.5 to 8.0 and no effect on the action potential height was noted.

B. Calcium-45 Efflux Study

A Ca-45 efflux experiment was done to investigate the possibility that an increase in hydrogen ion concentration may cause a release of

calcium from the nerve. Nerves were washed out in calcium-free Ringer's solution pH 7.2 for 30 minutes after which the pH of the medium was changed to pH 4.6. No increases in the rate of calcium-45 efflux was found when compared to control nerves which had been washed out in calcium-free Ringer's solution pH 7.2.

III The Effects of Choline Chloride Ringer's Solution and of Tetrodotoxin

A. Spontaneous Discharge and Action Potential Amplitude

Nerves which had been in calcium-free medium for 5 minutes and were spontaneously firing were placed in a calcium-free Ringer's solution containing 1×10^{-9} gm/ml (3×10^{-9} M) of tetrodotoxin (TTX). This concentration of TTX immediately abolished spontaneous discharge while the action potential was unaffected. Even after 40 minutes in TTX solution the amplitude of the action potential was unaffected (Fig. 15). Higher concentrations of TTX (1×10^{-8} gm/ml, 3×10^{-8} M) in calcium-free Ringer's solution abolished the evoked spike as well as spontaneous activity.

To investigate the effect of removing the external sodium on spontaneous discharge the action of choline chloride Ringer's solution was tested. When nerves were desheathed and placed in calcium-free choline chloride solution spontaneous activity did not occur. After 5 minutes the amplitude of the evoked spike was reduced to 23 per cent as compared to 45 per cent in control nerves in calcium-free Ringer's solution and gradually declined until it was abolished after 15 minutes. If nerves were returned to calcium-free Ringer's solution containing sodium, spontaneous activity

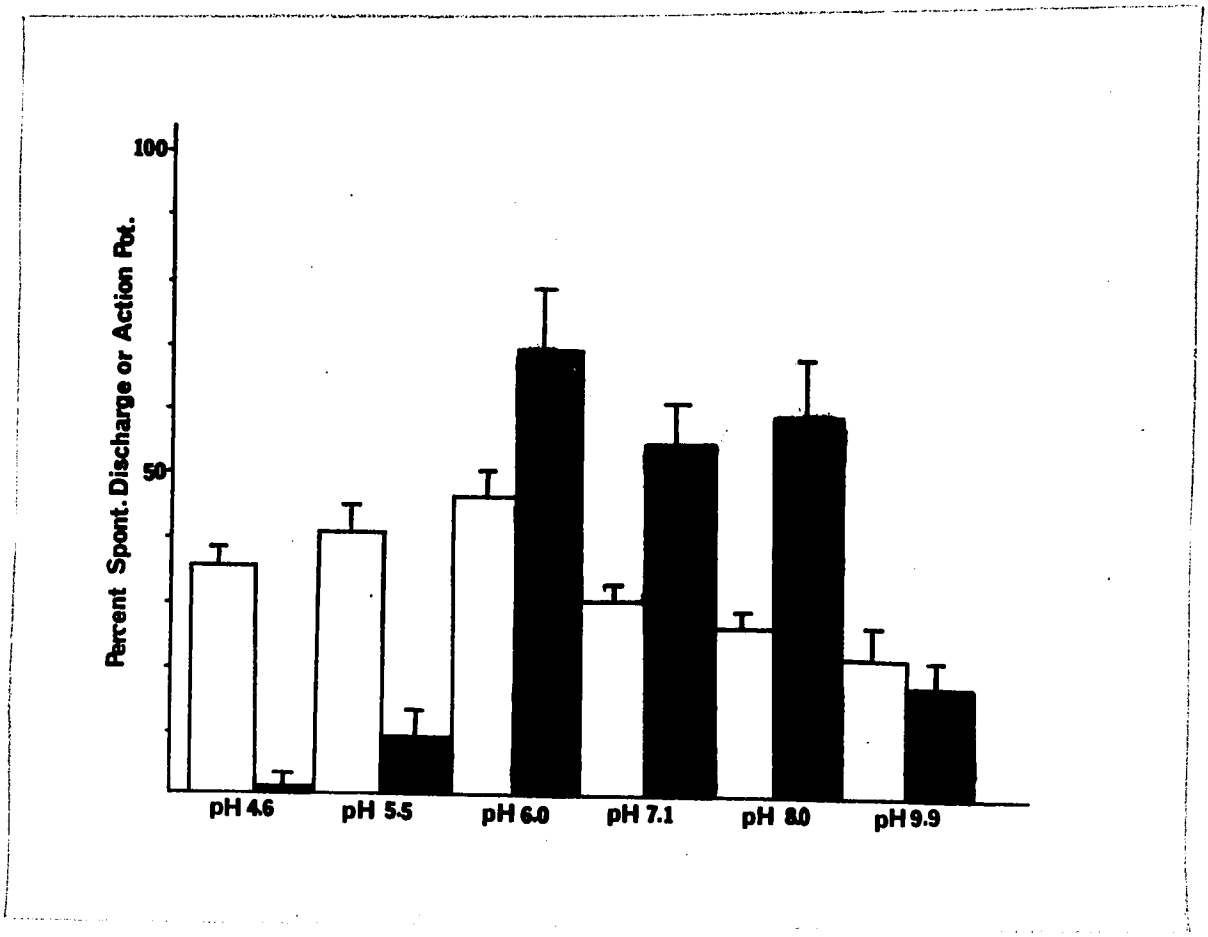

Fig. 16. Effect of pH on the per cent spontaneous activity (shaded bars) and the per cent action potential amplitude (white bars) in calcium deficient nerves after 25 minutes in each test pH. Bars are the mean of at least 6 separate experiments. The standard error ($\pm$SE) is given for each bar.

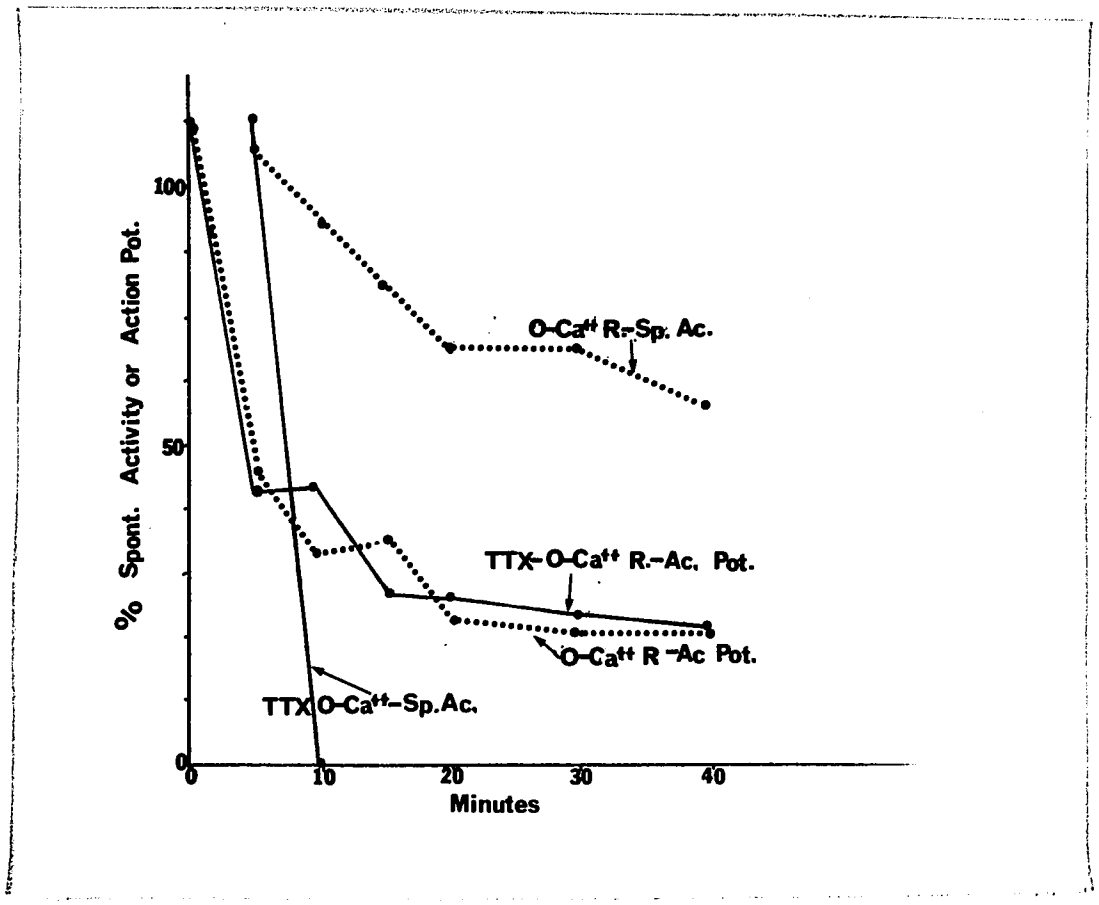

Fig. 17. Time course of action of 10^{-9} gm/ml of TTX (solid lines) in calcium-free Ringer's solution on the per cent spontaneous discharge and action potential amplitude in nerves previously exposed to calcium-free medium for 5 minutes. The time course of the action of calcium-free Ringer's solution (dotted line) on the per cent spontaneous activity and action potential amplitude is also shown. Each point is a mean of 6 separate experiments.

developed and the evoked spike was restored.

B. Calcium-45 Efflux Study

A calcium-45 efflux study was performed in which the nerves were first washed out in calcium-free medium for 30 minutes and then 1 x 10^{-9} gm/ml of TTX was added. No effect on the rate of calcium-45 efflux was found when compared to control nerves which were washed out in calcium-free Ringer's solution.

IV The Effects of Metabolic Inhibitors

A. Spontaneous Discharge and Action Potential Amplitude

1. 2, 4 Dinitrophenol (DNP)

A study was made of the action of 1 mM DNP in calcium-free Ringer's solution (pH 6.0)* on nerves which had previously been exposed to calcium-free medium for 5 minutes. Spontaneous discharge was abolished within 25 minutes of exposure (Fig. 18). At the time that spontaneous activity was abolished the height of the elicited action potential was equal to that of control nerves which had been soaked in calcium-free Ringer's solution (pH 6.0) for the same time interval. Spontaneous activity did not reappear in nerves which had been exposed to DNP in calcium deficient medium for 25 minutes and were returned to calcium-free Ringer's solution without DNP for 10 minutes. This indicated that the action of DNP on spontaneous

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* PH 6.0 was used in these experiments because it had previously been shown that at higher pH values DNP has little or no effect, presumably because it is more permeable at lower pH values (Shanes, 1958).

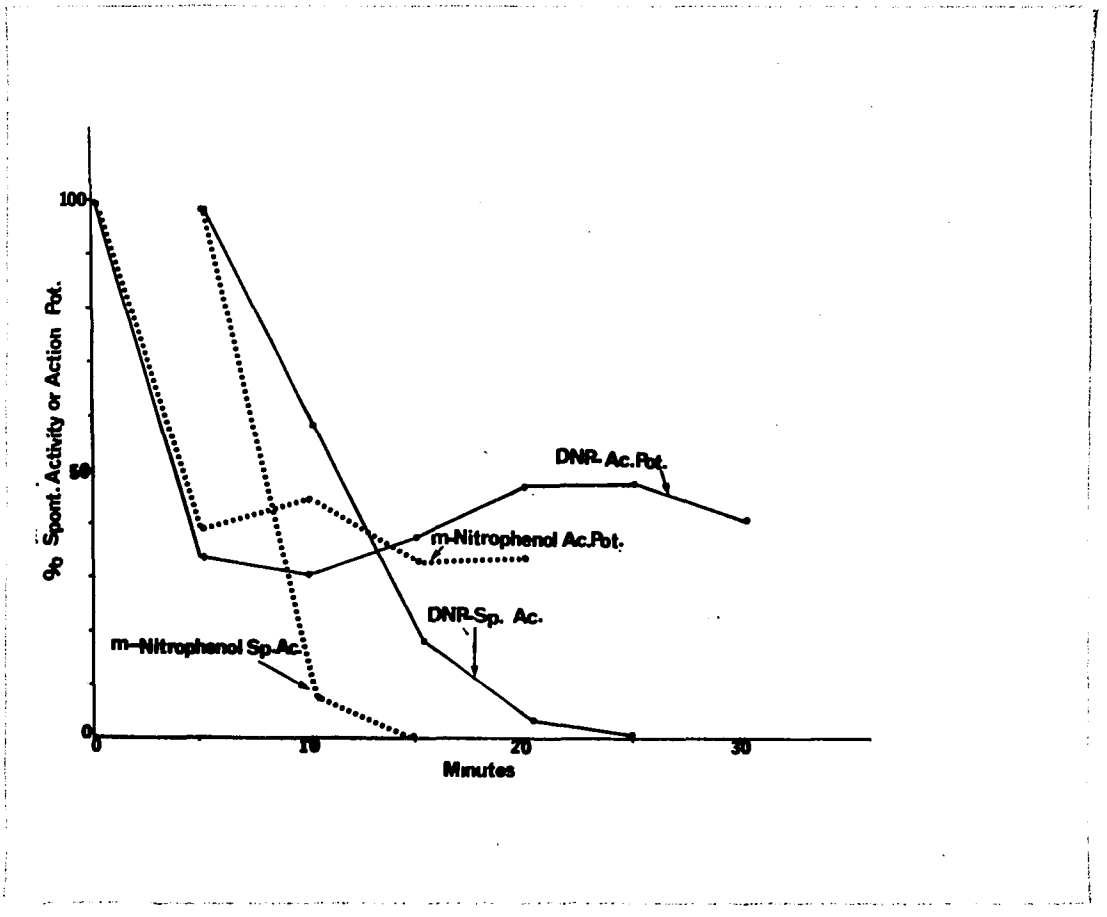

Fig. 18. The time course of the action of 1 mM DNP (solid line) and 1 mM m-nitrophenol (dotted line) in calcium-free Ringer's solution on the per cent spontaneous discharge and action potential amplitude in nerves previously exposed to calcium-free Ringer's solution for 5 minutes. Each point is a mean of 6 separate experiments. The standard error ($\pm$SE) is given for each point.

discharge may be irreversible. The antagonism of spontaneous discharge by DNP was studied at a number of concentrations and a regression line was determined (Fig. 19).

A series of experiments were done to investigate the effect of m-nitrophenol on spontaneous discharge. The results were similar to those found with DNP. At a concentration of 0.5 mM m-nitrophenol irreversibly abolished spontaneous activity in 20 minutes. The effect of 1 mM m-nitrophenol is shown in Figure 18. M-nitrophenol abolishes spontaneous discharge more rapidly than the same concentration of DNP.

In a second series of experiments desheathed nerves were first soaked in normal Ringer's solution containing 1 mM DNP at pH 6.0 for 25 minutes. (This soaking time corresponds to the time required for DNP to abolish spontaneous discharge in a calcium-free Ringer's solution.) The nerves were then placed in a calcium-free Ringer's solution and the spontaneous activity and action potential amplitude were measured. In control experiments nerves were exposed to a normal Ringer's solution at pH 6.0 for 25 minutes and then placed in calcium-free solution. The effect of the absence of sodium in the external medium during exposure to DNP on the ability of DNP to prevent spontaneous discharge was studied. Nerves presoaked in 1 mM DNP in choline chloride Ringer's solution, failed to develop spontaneous discharge while the control nerves which had been placed in choline chloride Ringer's solution for 25 minutes did develop spontaneous discharge when placed in calcium-free Ringer's solution. The action potential of nerves soaked in choline chloride Ringer's solution containing 1 mM DNP disappeared as did control nerves soaked

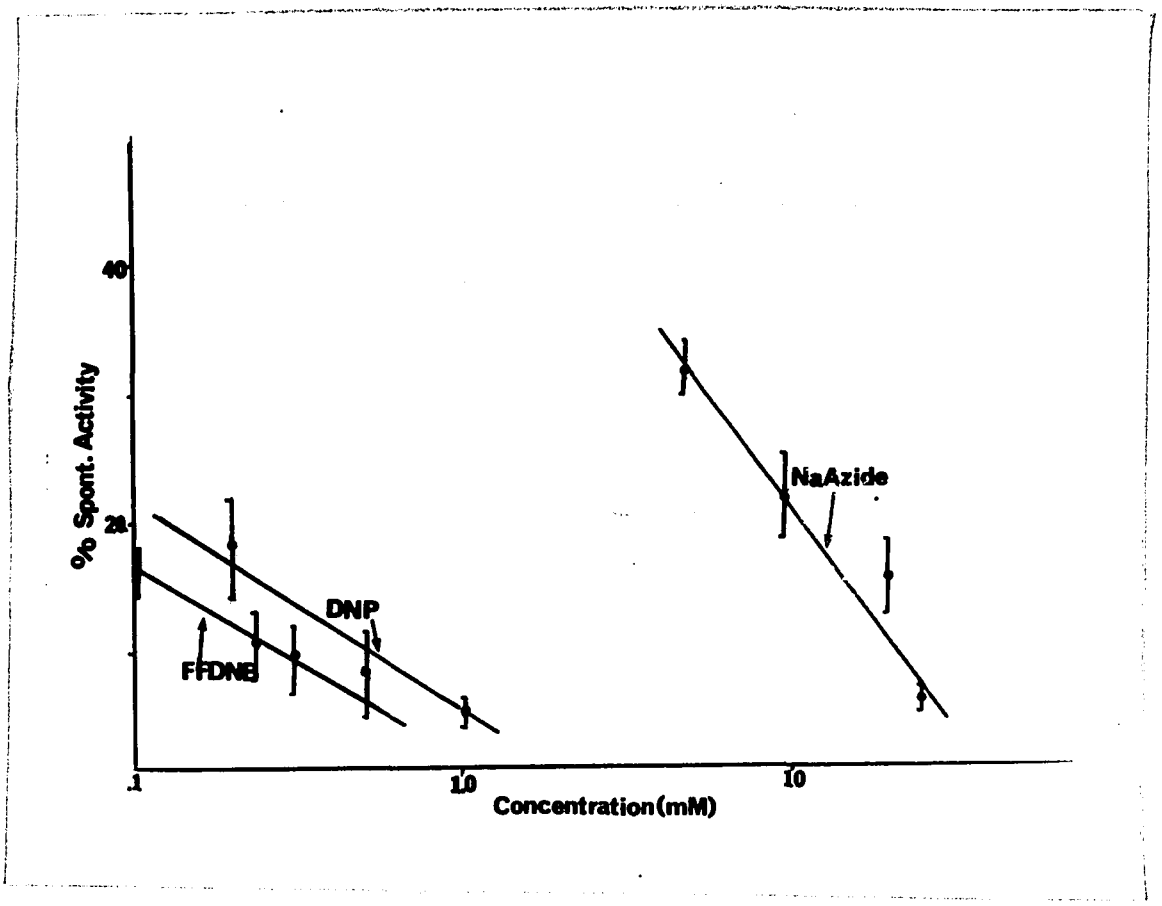

Fig. 19. Concentration-response regression lines for antagonism of spontaneous discharge by FFDNB, DNP and NaAz. Each point is a mean of at least 6 separate experiments. The standard error ($\pm$SE) is given for each point.

in choline chloride Ringer's solution but upon the addition of Na to the solutions the action potential reappeared.

2. Sodium Azide

The effect of sodium azide (NaAz), an inhibitor of the cytochrome chain, on spontaneous discharge was studied. Nerves which had previously been exposed to calcium-free Ringer's solution for 5 minutes and were spontaneously firing were placed in a calcium-free medium containing from 5.0 to 25.0 mM NaAz. Spontaneous activity was abolished within 30 minutes in 25 mM NaAz without a significant effect on the evoked spike. After 30 minutes NaAz was washed out but spontaneous activity did not reappear during the next 10 minutes. A dose response curve and regression line are shown in Figure 19. The slope of the regression line was significantly different from that of DNP ($P<0.01$).

3. Nitrobenzene Derivatives

The effect of several nitrobenzene derivatives on the calcium deficient nerve were tested. 1,5 difluoro 2,4 dinitrobenzene (FFDNB) (0.1 to 0.3 mM), 2,4 dinitrofluorobenzene (FDNB) (0.45 mM) and m-fluornitrobenzene (FNB) (3.0 mM) were all found to antagonize spontaneous activity caused by calcium deficiency. The dose response curve for FFDNB was made and a least squares regression line is shown in Figure 19. The slope was similar to that of DNP. Dose response studies of the actions of FDNB and FNB were attempted but results were impossible to interpret because of the rapid breakdown of these agents.

4. Other Agents

The ability of several other compounds to antagonize spontaneous discharge was investigated. Parachloromercuribenzoic acid (PCMB) and N-ethylmaleimide (NEM), both sulfhydryl reagents, were found to be nonspecific antagonists of spontaneous discharge since they significantly reduced the action potential amplitude at the time that spontaneous discharge was antagonized. As would be predicted the effects of both of these agents were prevented by the addition of equimolar concentrations of cysteine to the Ringer's solution. KCN (1 mM) was able to antagonize spontaneous activity within 30 minutes without affecting the evoked spike which was abolished only after 3 hours. Arsenic acid (10 mM), ouabain (1 mM) and carbonyl cyanide m-chlorphenyl hydrazone (65μm) were all nonspecific in their action, i.e., the action potential was also reduced.

B. Demarcation Potential Measurements

1. 2,4 Dinitrophenol (DNP)

Experiments were done to test the action of 1 mM DNP in both normal and calcium-free Ringer's solution on the demarcation potential. After equilibration in a normal Ringer's solution the section of nerve in compartment C was soaked in either a calcium-free Ringer's solution or a normal Ringer's solution containing 1 mM DNP at pH 6.0 for 25 minutes. For control experiments the solution in compartment C was replaced by calcium-free Ringer's solution at pH 6.0. The results (Fig. 20) indicate that the nerves which were exposed to 1 mM DNP in normal Ringer's solution gradually depolarized during the 25 minute exposure. At 20 minutes

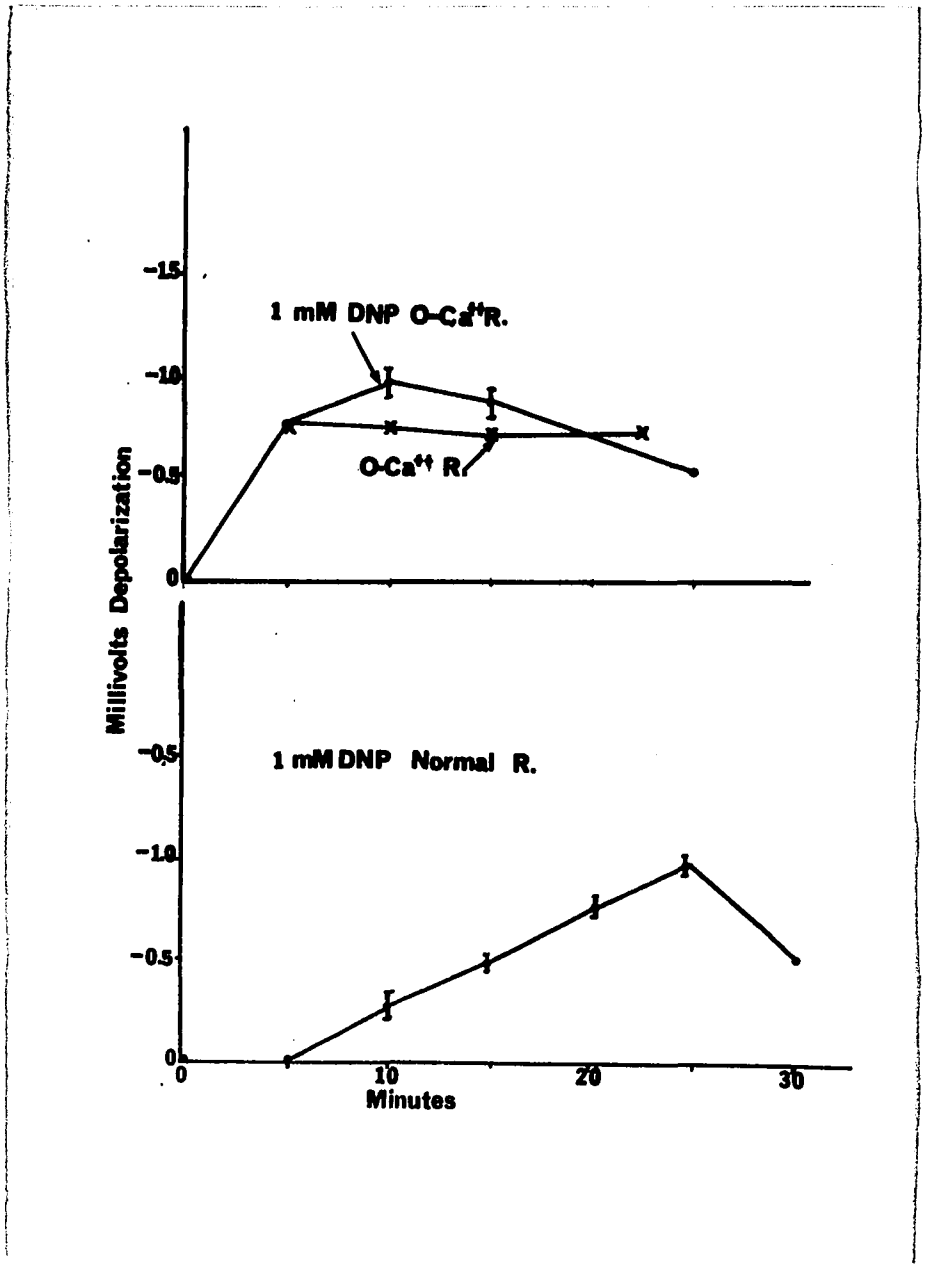

Fig. 20. The effect of 1 mM DNP in both calcium-free Ringer's solution ($0\text{-}Ca^{++}$) and normal Ringer's solution on the demarcation potential of frog nerves. Control nerves in calcium-free Ringer's solution (X's) are also shown. Each point is a mean of 6 separate experiments. The standard error ($\pm$SE) is given for each point.

the change in the demarcation potential was -1.0 ± 0.06 mv. The control nerves which had been placed in the calcium-free medium rapidly depolarized (within 2-3 minutes) reaching a maximum value of -0.75 ± 0.02 mv which was maintained for the 25 minute test period. During the first 5 minutes of the experiment the nerves which were placed in the calcium-free Ringer's solution containing 1 mM DNP had a greater depolarization (-1.0 ± 0.03 mv) than nerves in calcium-free Ringer's solution alone. The depolarization then decreased so that after 20 minutes it was actually at a lower level than that found in control nerves. From the data it is evident that DNP gradually depolarizes nerves in normal Ringer's solution but when nerves are exposed to calcium-free Ringer's solution DNP causes a transient increase in the depolarization normally seen.

The effect of 0.5 mM m-nitrophenol on the demarcation potential in calcium-free Ringer's solution and in normal Ringer's solution was also examined. M-nitrophenol did not significantly alter the demarcation potential.

2. Sodium Azide

Demarcation potential measurements were made on nerves exposed to 25 mM NaAz. Nerves which were placed in normal Ringer's solution containing 25 mM NaAz gradually depolarized reaching a value of -1.0 ± 0.04 mv after 20 minutes in the inhibitor (Fig. 21). Nerves which were exposed to 25 mM NaAz in calcium-free Ringer's solution also depolarized but this depolarization was much larger. After 20 minutes they reached a value of -1.6 ± 0.07 mv. This value is approximately equal to

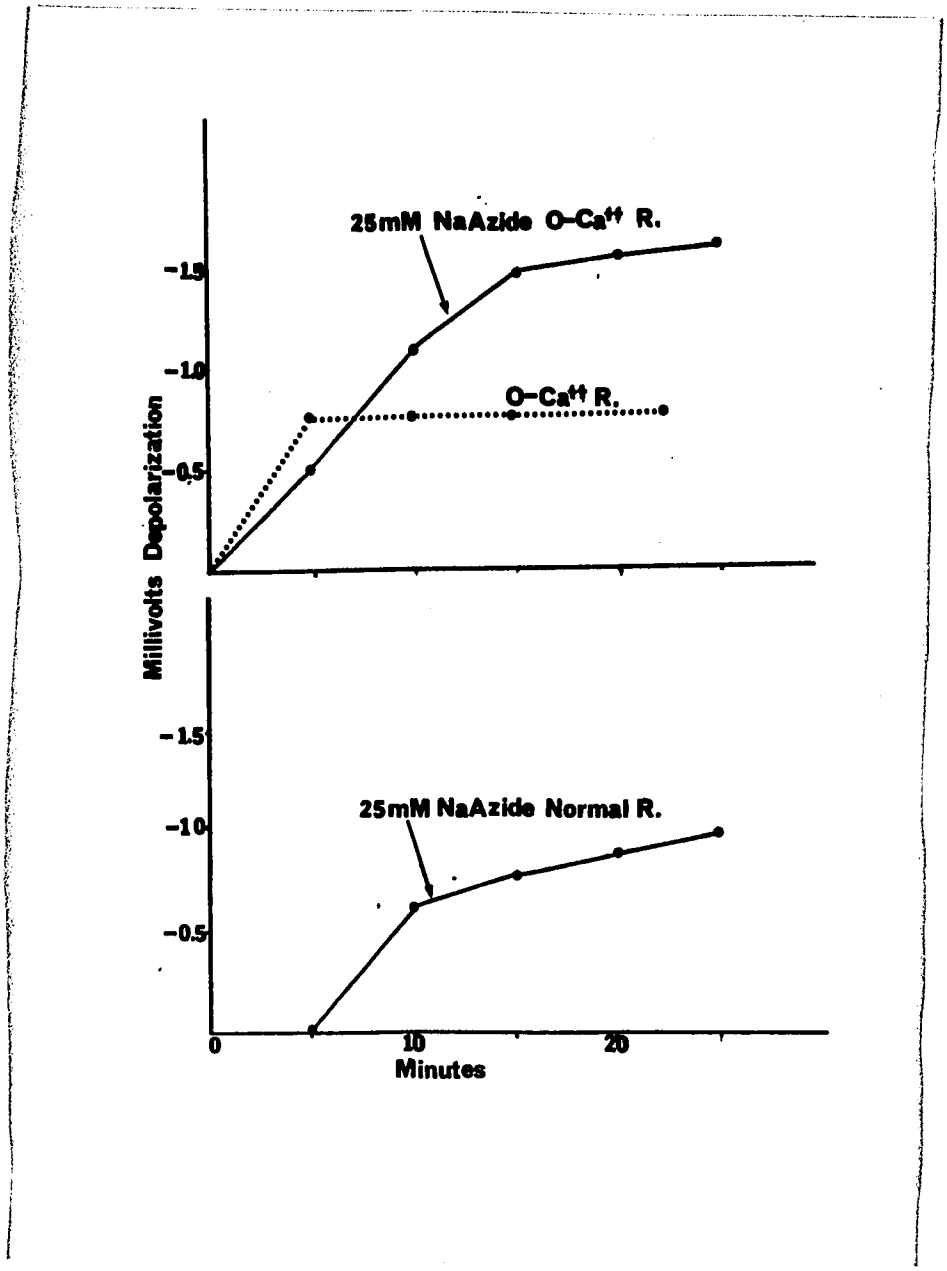

Fig. 21. The time course of the action of 25 mM NaAz in both calcium-free Ringer's solution and normal Ringer's solution on the demarcation potential of frog nerves. The time course of the action of calcium-free Ringer's solution (dotted line) on the demarcation potential is also shown. Each point is a mean of 6 separate experiments. The standard error ($\pm$SE) is given for each point.

the sum of depolarizations caused by 25 mM NaAz in normal Ringer's solution and that caused by calcium deficiency. It is evident that NaAz causes depolarization of nerve in both normal and calcium-free solutions.

C. Calcium-45 Efflux Studies

1. 2,4-Dinitrophenol (DNP)

It has previously been shown that DNP (1 mM) causes an increase in the efflux of calcium-45 into calcium-free Ringer's solution in the frog nerve (B. Altura, 1968). An efflux study was done to investigate the possibility that previous exposure of a nerve to DNP could effect calcium binding and therefore the calcium efflux when the nerve was placed in calcium-free Ringer's solution. This experiment was undertaken because the results presented earlier showed that presoaking the nerve in DNP will prevent spontaneous activity from developing in calcium-free medium. Nerves were washed out in 1 mM DNP normal Ringer's solution pH 6.0 for the first 30 minutes and then in a calcium-free Ringer's solution for the remainder of the test period. Control nerves were washed out in normal Ringer's solution pH 6.0 for the first 30 minutes and then calcium-free Ringer's solution was added. There was no detectable difference between the two sets of nerves.

2. Sodium Azide

Nerves were washed out in calcium-free Ringer's solution for 30 minutes and then exposed to a calcium-free solution containing NaAz (25 mM). Control nerves were exposed to calcium-free Ringer's solution for

the entire experiment. The results (Fig. 22) indicate that exposure to NaAz does increase the rate of calcium-45 efflux from the calcium deficient nerve and that this increase is maintai ned over the 50 minutes of the exposure.

D. ATP Measurements

The effect of DNP (1 mM) on the ATP content of the calcium deficient nerve was examined. Nerves which had been exposed to DNP pH 6.0 for 25 minutes in calcium-free Ringer's solution had statistically lower ATP content ($P<0.01$) than control nerves exposed to calcium-free Ringer's pH 6.0 for 25 minutes (Table 3a). Nerves exposed to 25 mM NaAz or 0.45 mM FFDNB for 25 minutes also showed a significant reduction in ATP content ($P<0.01$ and $P<0.01$ respectively) when compared to the control groups in calcium-free Ringer's solution (Table 3b-c).

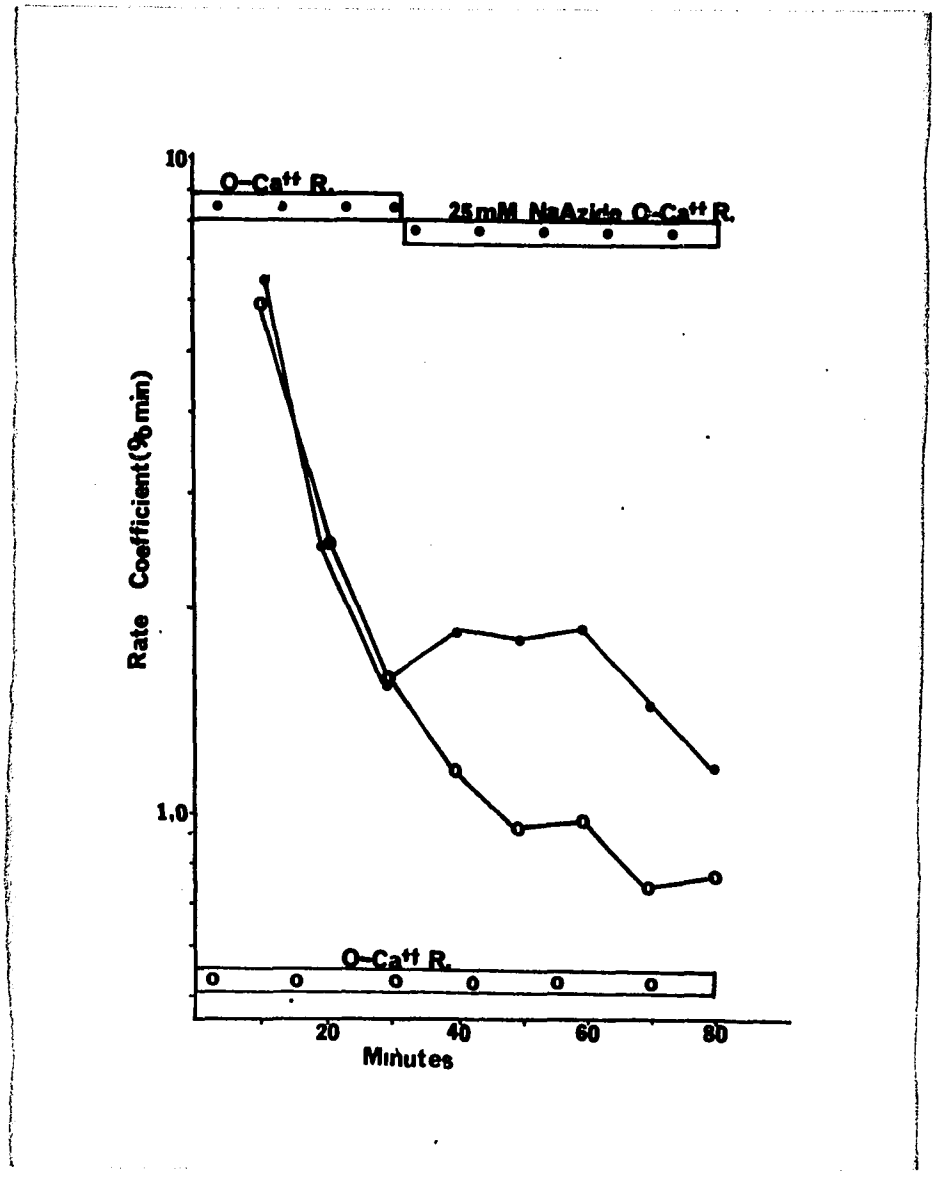

Fig. 22. The effect of 25 mM NaAz in calcium-free Ringer's solution on the rate coefficient of Ca-45 efflux in paired frog nerves. Experimental nerves (closed circles) were washed out in calcium-free Ringer's solution for the first 30 minutes after which NaAz was added. Control nerves (open circle) were washed out in calcium-free Ringer's solution for the entire experiment. The legends at the top and the bottom of the graph refer to the closed and open circles respectively. Each point is a mean of 3 separate experiments. Standard errors are given for the last half of the experiments.

Table 3. Effect of metabolic inhibitors on the ATP content of desheathed frog nerves soaked in calcium-free Ringer's solution for 25 minutes.

Exp. set	# pairs of nerves tested	Metabolic Inhibitor	uM ATP/gm wet wt.	Significance
a	9	Control	0.997 ± 0.05	P 0.01
		1 mM DNP	0.648 ± 0.10	
b	8	Control	0.934 ± 0.04	P 0.01
		25 mM NaAz	0.810 ± 0.04	
c	6	Control	1.11 ± 0.08	P 0.01
		0.45 mM FFDNB	0.819 ± 0.06	

DISCUSSION

The actions of compounds which selectively antagonize spontaneous discharge in calcium deficient desheathed sciatic nerves were studied. Compounds which depress both the action potential and spontaneous discharge are nonspecific antagonists. Nonspecific inhibitors comprise a wide variety of agents which cause inexcitability (including strychnine, iodoacetate, ethanol and many other poisons). Agents that antagonize spontaneous discharge at a time when the action potential amplitude is unaffected are considered selective antagonists. Spontaneous discharge can be selectively antagonized by increasing either the threshold of excitation or the membrane resistance. A decrease in either of these parameters facilitates spontaneous activity (Toman, 1952). Antagonists may be acting directly or indirectly on membrane sites which normally bind calcium, thereby increasing membrane resistance and/or threshold.

It has been proposed that calcium exerts its effects on the excitable membrane by binding to negative membrane sites (Hodgkin and Frankenhauser, 1957; Tobias, 1964; Koketsu, 1965). The results of the studies on antagonists of spontaneous discharge in calcium-free medium will be discussed with reference to their possible actions on these sites. It is postulated that the divalent cations tested, TTX and hydrogen ion are acting on these sites. The way they might influence calcium binding sites will be discussed. The action of a number of metabolic inhibitors was

also studied and it was proposed that they are acting through a metabolic effect which in turn influences excitability.

The interpretation of the action of the agents studied on the sciatic nerve is complicated by the fact that the measurements of both action potentials and spontaneous discharge involve the averaging of a large number of responses. The measurements made of spontaneous discharge are actually an average of a large number of unsynchronized action potentials and do not measure the underlying membrane changes involved in excitation (local oscillations). It is not possible to measure the frequency of the individual neurons which are firing in this experimental set up. Further experiments done to test the action of these antagonists on single fiber preparations would therefore be valuable in determining their site of action on the excitable membrane.

I Divalent Cations

The ability of a number of divalent cations to act as calcium substitutes was tested on the following parameters: inhibition of spontaneous discharge, repolarization of the calcium deficient nerve; release of Ca-45 from the nerve, and a change in the ATP content of the nerve. It was hoped that by determining which cations could substitute for calcium more could be learned about the role of calcium in nerve excitation and (more specifically) its binding sites on the membrane.

The transition metals that were tested (Mn, Co, Ni, Zn) were more effective antagonists of spontaneous discharge than the alkali earth metals (Ba, Sr, Mg). Barium, in fact, did not completely antagonize

spontaneous discharge even at a concentration of 1.8 mM. This is surprising since other workers measuring Na and K conductances and the rate of rise of the action potential have found that barium is a very effective calcium substitute in the lobster axon (Blaustein and Goldman, 1968 and Hafemann, 1969a). The discrepancy between the action of barium as an antagonist of spontaneous activity in the desheathed frog nerve and its effects on the lobster axon may result from the fact that calcium deficiency is not identical in the two systems - i.e., the lobster axon does not always exhibit spontaneous discharge and depolarization as does the frog nerve (Adelman, 1956). It also must be noted that different parameters were measured in these studies and it is possible that different mechanisms are operative in each case. Both Blaustein and Goldman (1968) and Hafemann (1969) found that the transition metals could also substitute for calcium in the lobster axon, although Hafemann reported that axons became inexcitable after 30 minutes in solutions containing transition metals.

A number of workers have postulated that calcium binding to negative phospholipid and protein sites on the membrane plays an important role in excitation (Tobias, 1964, Koketsu, 1965, Adelman and Dalton, 1960). The cations which are antagonists of spontaneous discharge are probably acting by either binding to negative membrane sites or by indirectly affecting the binding of calcium to these sites. Some properties of the cations tested are listed in Table 4. There is a correlation between the cations that are the most effective antagonists of spontaneous discharge and their ionic radii. Of the cations with ionic radii smaller than calcium, those most effective have ionic radii most similar to calcium. Strontium

and barium which are very weak antagonists, have ionic radii which are larger than calcium.

The best antagonists of spontaneous discharge (Co, Mn, etc.) bind more strongly to N, S and O ligands than other cations (Martell, 1961). Mn, Co, Ni and Zn prefer nitrogen ligands while Ca, Ba, Sr and Mg prefer oxygen ligands (Williams, 1961). Blaustein and Goldman (1968) postulate that the differences they observed between the effects of cations on the lobster axon were probably due to differences in the rate constants of dissociation and association from membrane sites. The results from the experiments on the desheathed frog nerve may also be interpreted in this way. The fact that Co, Mn and Ni bind more strongly to both O and N ligands would mean that they dissociate more slowly than calcium from these sites. This would inhibit spontaneous discharge if the dissociation of a divalent cation from membrane sites was involved in spontaneous discharge. Several workers have proposed that the dissociation of a divalent and the association of a monovalent cation is necessary for excitation (Tobias, 1964 and Koketsu, 1965).

By measuring the demarcation potential in the calcium deficient nerve, a correlation was found between the ability of a cation to antagonize spontaneous discharge and its ability to repolarize the nerve. It is noteworthy that barium did not repolarize the nerve, and in fact caused a slight depolarization. Nishi, Soeda and Koketsu (1965) noted that barium also caused a depolarization in the toad spinal ganglia. They concluded that barium has a sodium like action, in that it can carry the current. It has been shown that frog sympathetic ganglia cells will conduct action

Table 4. Chemical properties of divalent cations tested for their ability to antagonize spontaneous discharge.

Cation	Relative Effectiveness in Antagonizing Sp. Discharge	Ionic* Radius (A°)	Preferred* Ligand	S/O*	N/O*
Ca	1.0	0.99	O	-1.5	0.0
Mn	1.24	0.78	N	+1.9	+2.1
Co	1.69	0.74	N	+4.1	+3.9
Ni	2.9	0.73	N	+2.4	+4.4
Zn	4.1	0.72	N	+6.3	+3.6
Mg	5.3	0.66	O	-1.1	+0.9
Sr	5.3	1.15	O	-1.4	-0.2
Ba	-	1.37	O	-1.2	-0.4

*Hafemann, 1969

potentials in isotonic $BaCl_2$ (Koketsu and Nishi, 1969).

The results of calcium-45 efflux studies on the sheathed nerve have shown that Sr and Ba release almost as much calcium as calcium itself while Mn and Co are very poor calcium releasers (Altura, 1968). Experiments on the desheathed nerve agree with these findings. There appears to be no correlation between the ability of a cation to antagonize spontaneous discharge and its ability to release calcium. This may be due to the difficulty in detecting a fraction of calcium which is related to spontaneous activity. Spontaneous activity occurs within 5 minutes after immersion in calcium-free medium; therefore the calcium that is being released and which is important in the maintenance of nerve function may be coming out during the first 5 minutes in calcium-free Ringer's solution. If nerves are treated with calcium-free medium immediately after loading with calcium-45 the efflux during the first 20 minute period is very large, the bulk is probably coming from the extracellular space (Soloway, et al., 1953, Altura, 1968). Thus any cellular fraction washed out at this time would be masked by calcium coming from the extracellular space.

To eliminate this problem experiments were done in which nerves were washed out in normal Ringer's solution for 30 minutes prior to adding calcium-free solution. Figures 11, 12 and 13 show the results of these experiments. These results indicate that Sr acts like calcium itself (causing the same decrease in tissue calcium as nerves washed out in normal Ringer's solution) while manganese is a poor calcium releaser (causing a slight increase in tissue calcium as compared to nerves soaked in normal Ringer's solution). These results are in agreement with the

previous findings although the differences between the effects of Sr and Mn are very small in this case. This is probably due to the fact that washing the nerves out in normal Ringer's solution for the first 30 minutes of the experiment enables some of the cell calcium to exchange for unlabelled calcium by the time calcium-free Ringer's solution is added.

Unfortunately the results of these experiments cannot be interpreted as representing differences in the ability of calcium to exchange with Sr and Mn at calcium binding sites since the flux may still be coming from extracellular sites. The fact that the rate of calcium-45 efflux is changing with time throughout the entire washout experiment indicates that the calcium is coming from a heterogenous compartment (which may include any number of sites, intracellular or extracellular). If the flux studies could have been carried out for longer (unfortunately the nerves begin to degenerate) a time may have been reached in which the rate of efflux from nerves washed out in calcium-free Ringer's solution would become constant and the flux from control nerves in normal Ringer's solution would approach zero. The difference in these two curves would then represent bound calcium and the effects of other agents on this fraction could be studied.

Although no quantitative conclusions can be reached about the effects of Sr and Mn on the bound Ca fraction the results do agree with previous experiments indicating that Sr is a better calcium releaser than Mn. There may be several reasons why the effects of divalent cations on calcium-45 efflux do not correlate with their ability to antagonize spontaneous discharge. It is possible that the fraction of calcium which is

important is not being detected because it is lost too quickly or because it comprises too small a fraction of the total exchangeable calcium fraction to be measured. A factor which may also complicate the interpretation of the calcium-45 efflux data is whether the radioactive calcium was completely equilibrated with the total tissue calcium at the time the washout experiments were begun. Actual measurements of equilibration for nerves soaked in 10 ucuries/ml of Ca-45 for 3 hours at room temperature were not made but rough estimates were calculated to be 34 per cent.* The nerves were therefore not equilibrated when the washout experiments were done. It is possible that the lack of correlation between the flux data and the electrical data is due to the fact that a compartment of calcium which is important in antagonizing spontaneous discharge is not sufficiently labelled and therefore is not being detected. This is unlikely because the calcium which is critical in controlling spontaneous discharge is a rapidly exchanging fraction and therefore should be labelled within a few minutes. The most probable reason for the lack of correlation between

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* The per cent equilibration = $\frac{\text{Specific Activity of nerve}}{\text{Specific Activity of medium}} \times 100$.
The specific activity of the medium = $\frac{\text{CPM in medium}}{\text{Total Ca conc.}}$ or
$\frac{(10 \text{ ucuries})(222 \times 10^4 \text{dpm})(0.76 \text{ cpm/dpm})}{1.8 \text{ umoles}}$. The values for both the cpm in the external medium and the total calcium concentration were not directly measured and had to be estimated. The specific activity of the medium was 89.3×10^5 cpm/umole. The specific activity of the nerve = $\frac{\text{CPM in nerve}}{\text{Total Ca in nerve}}$ or $\frac{61 \times 10^5 \text{ cpm/gm tissue}}{2 \text{ umoles}} = 30.5 \times 10^5 \frac{\text{cpm}}{\text{umole}}$.
The value for the total nerve calcium was not measured directly on the nerves tested but was taken from previous measurements in this laboratory (Altura, 1968). The per cent equilibration of the nerves after soaking in 10 ucuries/ml of Ca-45 for 3 hours was therefore approximately

$$\frac{30.5 \times 10^5}{89.3 \times 10^5} \times 100 \text{ or } 34\%.$$

the effects of the cations tested on flux measurements and their ability to antagonize spontaneous discharge is that only a small labile fraction of calcium is involved in controlling spontaneous discharge and this fraction is not being detected.

Gerard (1932) and Abood (1966) have suggested a connection between metabolism and calcium. Abood, et al. (1962) found that there was a considerable increase in the efflux of orthophosphate and ATP from bullfrog nerves exposed to either calcium-free or potassium rich medium. The finding that the ATP content in the desheathed nerve decreases after a 25 minute exposure to calcium-free medium may be related to an increased efflux of ATP. It is possible that the decreased ATP content is due to a more direct effect of calcium on a metabolic pathway. It may simply be due to increased nerve activity during spontaneous activity. Brink, et al. (1946) showed that calcium deficiency increased the oxygen consumption of nerves. The finding that nerves exposed to 3 mM KCl Ringer's solution did not exhibit a decreased ATP content indicates that the decrease in ATP content in the calcium deficient nerve is not solely caused by the depolarization associated with calcium deficiency. Abood (1964) found that 120 mM KCl lowered the ATP content in bullfrog nerves by 18 per cent but that 60 mM KCl had no effect. A very large depolarization by KCl is required to lower the ATP content in nerves.

The decreased ATP content in nerves soaked in calcium-free medium may be interpreted in terms of Abood's (1965) theory. He proposes the existence of a Ca-ATP complex in the membrane; depolarization is associated with calcium release from the membrane, and rupture

of the Ca-ATP complex. These events in turn cause a structural modification in the membrane that somehow leads to an impulse. According to this view, it is possible to see how calcium deficiency, by increasing the dissociation of calcium from membrane sites, could enhance the breakdown of ATP and therefore lower the ATP content of the nerve. It has been shown by Okamoto, et al. (1964), that ATP can antagonize spontaneous activity in the calcium deficient desheathed frog nerve. That ATP is not acting here solely by chelating calcium is obvious since it has been shown in this laboratory that other calcium chelators (e.g. EDTA) do not antagonize spontaneous activity. It is possible that the loss of both ATP and calcium may be necessary for spontaneous activity to develop, and if either is replaced spontaneous activity ceases.

The findings that the addition of 1.8 mM $MnCl_2$ or $CoCl_2$ to calcium deficient medium does not prevent the decrease in ATP content in calcium deficient nerves indicates that these cations cannot substitute for calcium in this case. This indicates that the loss of ATP in a calcium deficient nerve may be unrelated to spontaneous repetitive discharge. The addition of 1.8 mM $SrCl_2$ further decreased the ATP content of the nerve. This may be related to the fact that addition of strontium causes an increased release of calcium in calcium-free medium (Ca-45 efflux data); if at least some of this calcium is complexed to ATP, it then follows that ATP would also be released.

II Hydrogen Ion

Experiments performed to test the effects of changes in hydrogen ion concentration on spontaneous discharge and the action potential amplitude

indicated that spontaneous discharge was more sensitive to low pH than the action potential. At a pH between 5.5 and 4.5 spontaneous discharge is completely antagonized while the action potential is only slightly affected. Hydrogen ions probably act on negative membrane sites which are important in maintaining spontaneous discharge. Hille (1968a) studied the effects of changes in hydrogen ion concentration on the voltage clamped frog node of Ranvier. He found that low pH caused a positive shift of the voltage dependent parameters of the Hodgkin-Huxley model and also decreased the sodium conductance. The antagonism of spontaneous discharge by low pH is compatible with these findings. Hille suggested that the decrease in sodium conductance was due to the protonation of an acidic group (with a pKa of 5.2) that was necessary for the functioning of the sodium channel.

The fact that the action potential is not affected at a pH which antagonizes spontaneous discharge could be explained if some calcium still remained on acidic membrane sites protecting them from the increased hydrogen ion concentration. The sites which are important in controlling spontaneous discharge in calcium-free medium probably no longer have calcium bound to them and are therefore protonated at low pH. Hafemann (1969b) showed that high calcium could protect against the effects of low pH in the lobster axon.

III Tetrodotoxin and Choline Chloride Ringer's Solution

Spontaneous discharge in calcium-free Ringer's solution is suppressed by TTX (10^{-9} gm/ml) without any effect on the height of the evoked spike. TTX has been found to block the flow of cations through the transient

channel in squid axon (Moore, et al., 1967), lobster axon (Narahashi, 1964) and the frog node of Ranvier (Hille, 1968). The effects of TTX on spontaneous discharge can easily be explained by its action on the early transient current. That spontaneous activity is antagonized at a concentration which does not affect the evoked spike indicates a difference in sensitivity to TTX. It is possible that TTX is affecting the same membrane sites to which calcium normally binds and thereby antagonizes spontaneous discharge. A number of investigators have shown that the active form of TTX is the cationic form (Camougis, et al., 1967, Hille, 1968b). The finding that the concentration of TTX which abolishes spontaneous discharge does not affect the evoked spike could be explained if fewer negative membrane sites need to be bound by TTX for spontaneous discharge to be antagonized than for the action potential to be abolished. Only a percentage of membrane calcium is probably lost in calcium-free medium. The lost calcium might by changing membrane configuration cause the further loss of calcium, increasing membrane permeability and causing excitation. The binding of TTX to these sites in calcium-free medium could antagonize the resulting excitation. During the evoked spike the initial stimulus probably causes the release of more membrane calcium so that more negative sites must be blocked by TTX.

It has been proposed that hydrogen ions are affecting the same negative sites as TTX (Hille, 1968b). Both of these cations affect the sodium channel and it is possible that they are acting on an acidic group that is part of this channel. It is interesting that both agents are capable of antagonizing spontaneous discharge before the evoked spike is affected.

The experiments with choline chloride Ringer's solution indicate

that the presence of sodium in the external solution is necessary for spontaneous discharge to occur. This is expected since the entry of sodium or some other cation is needed for excitation. Wright and Tomita (1965) reported a requirement of 3.3 Na to 1 Ca in the external medium for spontaneous activity in the lobster axon. It takes about 10 minutes to abolish the evoked spike in sodium-free Ringer's solution while spontaneous discharge is immediately antagonized. This delay may be caused by the presence of some sodium in the extracellular space.

IV Metabolic Inhibitors

It has previously been reported that DNP and azide antagonize spontaneous discharge in the frog nerve (Altura, 1968). Other metabolic inhibitors tested which selectively antagonized spontaneous discharge were fluoronitrobenzene derivatives, m-nitrophenol and KCN. All of these agents can inhibit metabolism. DNP and m-nitrophenol are uncouplers of oxidative phosphorylation, KCN and NaAz inhibit respiration and fluornitrobenzene derivatives covalently bind to proteins and certain lipids to form stable derivatives (Cooke, et al., 1969), thereby inactivating enzymes.

It is tempting to propose that these agents are acting by inhibiting the Na-K pump which maintains the resting potential in nerves since this is the only known link between metabolism and excitability (Hodgkin, 1964). Using radioactive tracers Hodgkin and Keynes (1955) showed that DNP, azide and cyanide inhibited the Na-K pump in lobster and squid axons. These agents also abolish post tetanic hyperpolarization in nonmyelinated nerve fibers (den Hertog and Ritchie, 1969). However, it is difficult to

explain antagonism of spontaneous discharge by these agents through their effect on the Na-K pump without a parallel action on the evoked spike. Inhibition of the Na-K pump should lead to the passive dissipation of the sodium and potassium gradient and therefore to inexcitability without selectively abolishing spontaneous discharge. If the accumulation of sodium inside or potassium outside the axon is responsible in some way for the antagonism of spontaneous discharge a change in the resting potential should be seen. Although NaAz does have a depolarizing action on nerves, DNP only slightly depolarizes the nerve and m-nitrophenol has no detectable effect. Spontaneous discharge in sodium-free medium should not be inhibited by these agents if the accumulation of sodium within the fibers was involved. Nerves presoaked in choline chloride Ringer's solution containing 1 mM DNP failed to develop spontaneous discharge just as those presoaked in normal Ringer's containing 1 mM DNP even though oscillatory behavior may still be present. Moreover, ouabain which interferes with the active transport of sodium in nerves (Skou, 1965) antagonizes both spontaneous discharge and the evoked spike at the same time. If an effect on the Na-K pump were the mechanism by which all the other inhibitors were acting ouabain would probably be a selective antagonist of spontaneous discharge also. Therefore, a different interpretation of the action of these agents is necessary.

It has been reported in squid axons (Segal, 1968) and frog nerves (Schoepflie and Bloom, 1959) that metabolic inhibitors affect excitability through a metabolic action that does not involve the Na-K pump. Segal (1968) investigated the effects of DNP and cyanide on the survival time of excitability in squid axons and concluded that these agents were not acting

only by inhibiting the Na-K pump but have a more direct action on metabolism since they are effective in sodium-free medium. These investigators also found that axons placed in solutions containing ouabain survived twice as long as those in metabolic inhibitors. Schoepflie and Bloom (1959) proposed that CN and DNP depressed the sodium conductance in the frog nerve through an action on oxidative metabolism. They found that single nerve fibers exposed to these agents exhibited a 10 per cent reduction in spike height after 30 minutes. These findings disagree with the results of the present studies on the effects of cyanide and DNP on the desheathed sciatic nerve. Schoepflie and Bloom (1959) found a 10 per cent reduction in spike height with these agents. This reduction is very small and may not be significant since controls and standard errors were not shown. Experiments on the ability of these agents to depress spontaneous discharge were performed on nerves in calcium-free solution. Therefore the spike height was reduced even before the inhibitor was added. It is possible that the depression caused by calcium deficiency masked any slight effect that may have been seen during this time period.

The possibility exists that a metabolic effect is not involved and that these agents antagonize spontaneous discharge through a direct action on either axoplasmic or membrane sites which control ionic changes during spontaneous activity. Cooke, et al. (1968), studied the action on nitrobenzene derivatives on the squid and lobster axons. FFDNB and FDNB abolish action potentials in these axons in 20-30 minutes without affecting the resting potential or membrane resistance. It was concluded that these agents act primarily on the cation permeability changes underlying excitation

and secondarily on the resting potential or the metabolism of the nerve because other known metabolic inhibitors (i.e., cyanide) do not affect the action potential of these axons (Hodgkin and Keynes, 1955). This type of reasoning cannot be used in the present case since metabolic inhibitors such as DNP, azide and cyanide as well as fluoronitrobenzene derivatives inhibit spontaneous discharge. It seems unlikely that these compounds which are all very different chemically could produce a similar chemical reaction on critical sites. It is much more likely that they are acting by inhibiting metabolic pathways which are necessary for the maintenance of spontaneous discharge, although the possibility that any number of them exert a more direct action on the nerve cannot be excluded.

At least a 20-30 minute exposure to each of these agents is necessary to completely antagonize spontaneous discharge. Divalent cations antagonize spontaneous discharge within 1-2 minutes. The slow action of these inhibitors implies an indirect effect although it is possible that they are acting directly on axoplasmic sites and permeability barriers prolong their action. It is unlikely that they are acting directly on membrane sites.

The mechanism of action of these inhibitors may involve a release of calcium. DNP, azide and cyanide release calcium from the squid axon (Rojas and Hidalgo, 1968) and from the frog nerve (Altura, 1968). Blaustein and Hodgkin (1969) postulate that the release of calcium from the squid axon by cyanide may be caused by a release of intramitochondrial calcium flooding the axoplasm. The metabolic inhibitors tested in this system may act on oxidative metabolism in such a way that calcium is released from mitochondria increasing membrane calcium and therefore inhibiting spontaneous

discharge. The efflux study in which nerves were first washed out in normal Ringer's solution with DNP (1 mM) and then calcium-free Ringer's solution showed that presoaking in DNP did not affect the calcium efflux into calcium-free medium. If these agents were acting to release intra-axonal calcium an increase in the calcium efflux might be seen.

ATP measurements show that NaAz (25 mM), DNP (1 mM) and FFDNB (.45 mM) all significantly decreased the ATP content of frog nerves within the same time period that spontaneous discharge was inhibited. Whether these two phenomena are directly related cannot be definitely decided on the basis of the experiments. It could be argued as unlikely that the loss of ATP can inhibit spontaneous discharge since the addition of exogenous ATP to the medium effectively antagonizes spontaneous activity. But exogenous ATP may have quite a different effect on the nerve than internally produced ATP. The decrease in ATP content in nerves exposed to metabolic inhibitors could be due in part to the release of calcium caused by these agents. If calcium and ATP are linked together on the membrane as Abood (1965) suggests then the release of one of these may in turn release the other.

In summary, the antagonism of spontaneous discharge by these inhibitors may be caused by either an effect on metabolism or a direct chemical interaction with axoplasmic sites. It does not, however, appear to be by direct action on the membrane. It is postulated that a metabolic effect is involved because of the long time course of action of these agents and the variety of different inhibitors which are effective. Gerard (1932) has suggested that calcium links electrochemical processes to metabolism in nerves.

If so, loss of calcium, which increases excitability, may also significantly activate metabolic pathways. This activation may be a requisite for spontaneous activity. If this is true the addition of metabolic inhibitors to a spontaneously active nerve would antagonize spontaneous discharge by inhibiting these pathways. Brink, et al. (1946) found that the addition of NaAz to calcium deficient nerves abolishes spontaneous activity as well as preventing the increased oxygen consumption which occurs during calcium deficiency. They therefore concluded that an increased metabolic rate as well as certain chemical changes are necessary for the initiation of spontaneous discharge. Spontaneous discharge in frog nerve is also very sensitive to changes in temperature, having a temperature optimum of 24°C (Altura, et al., 1967). This supports the hypothesis that metabolic changes may be directly involved in the occurrence of spontaneous discharge. It is possible that metabolism is more involved in excitatory processes than is generally appreciated.

SUMMARY

1. Selective antagonists of calcium-free spontaneous discharge were studied in the desheathed frog sciatic nerve to clarify the role of calcium in stabilizing the nerve and the characteristics of the calcium binding sites involved. The effects of these agents on the action potential amplitude, demarcation potential, calcium-45 efflux and ATP content of nerves were examined.

2. Divalent cations tested antagonize spontaneous discharge in the following order: Ca > Mn > Co > Ni > Zn > Mg, Sr. Cations that were the most effective antagonists have ionic radii most slimiar to that of calcium and also have greater binding constants for oxygen, nitrogen and sulfur sites. Demarcation potential studies indicated that cations which antagonized spontaneous discharge also repolarized the calcium deficient nerve. Calcium-45 efflux data indicated that the best calcium releasers were not the most effective antagonists of spontaneous discharge. The lack of correlation between these two parameters is discussed. Calcium deficiency also decreased the ATP content of nerves. This is most probably related to an action on nerve metabolism. The decrease is not prevented by divalent cations which are good antagonists of spontaneous discharge.

3. Low pH antagonized spontaneous activity before the evoked spike was affected. The pKa of the acidic group involved was between 4.5-5.5. Low pH did not release calcium from the nerve. Antagonism of spontaneous

discharge by hydrogen ions is most likely due to binding of hydrogen ions to negative membrane sites which normally bind calcium.

4. TTX (10^{-9}gm/ml) antagonized spontaneous discharge at a concentration that had no effect on the spike height. This action is explained by the inhibition by TTX of early transient currents. Choline chloride Ringer's solution inhibited spontaneous activity as would be expected if sodium ions were carrying the current.

5. Metabolic inhibitors which selectively antagonized spontaneous discharge were: DNP, KCN, NaAz, m-nitrophenol, FFDNB, FDNB, FNB. These agents probably did not act by depolarizing the nerve since only NaAz caused a significant depolarization. DNP, NaAz and FDNB decreased the ATP content in the nerves tested. These inhibitors most likely act indirectly on membrane sites through metabolic effects. They may affect these sites through the release of calcium or the decrease in ATP content.

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